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Combinatorial Therapy of Doxorubicin and MDR1 SiRNA by Polymeric Micellar Nanoparticles in Treatment of Multidrug Resistance in Breast Cancer

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COMBINATORIAL THERAPY OF DOXORUBICIN AND MDR1 SIRNA BY POLYMERIC MICELLAR NANOPARTICLES IN TREATMENT OF MULTIDRUG RESISTANCE IN BREAST CANCER

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ABSTRACT

Despite tremendous progress within the field of oncology, highly metastatic forms of breast cancer remain particularly challenging to effectively treat. Systemically delivered chemotherapy with cytotoxic agents typically requires some convalescence time between treatments – allowing rapidly growing cancer types to develop resistance. Multidrug resistance, also known as pump-dependent, is particularly difficult to treat as it functions through overexpression of P-glycoprotein, an efflux pump which can produce resistance to a range of chemotherapeutics.

We have previously developed the cationic micellar copolymer poly(lactide-co-glycolide)-g-poly(ethylenimine) (PgP) and demonstrated its capacity as a vector for gene therapy. Here, we examine the capacity of PgP in mediating co-delivery of siRNA targeting P-glycoprotein (siMDR1) and the anthracycline class drug doxorubicin to mitigate multidrug resistance in drug resistant triple-negative human cancer cells *in vitro*. The results of this project have shown that PgP can be used to successfully bind siRNA into a complex that protects from interaction with charged particles through heparin competition assay, and remains stable in serum conditions. Results of MTT assay assessing metabolic activity have shown PgP/siMDR1 complexes to exhibit minimal cytotoxicity *in vitro* in comparison to untreated human MDA-MB-435 cancer cells. Assessment of silencing following treatment with PgP/siMDR1 complexes formed at various nitrogen:phosphate (N/P) ratios has shown significant knockdown of MDR1 mRNA up 67%, in comparison to untreated groups of drug resistant MDA-MB-435 cells. PgP has also been shown to successfully load the hydrophobic chemotherapeutic

doxorubicin, improving the toxicity of the drug *in vitro*. These results show the efficacy of PgP as a vehicle for delivery of both doxorubicin and siMDR1 to drug resistant cancer cells, and may have potential for use in co-delivery of siMDR1 and chemotherapeutics in metastatic cancer treatment. Future studies will include *in vivo* toxicity and antitumor studies in athymic mouse breast cancer models.

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CHAPTER ONE

INTRODUCTION & BACKGROUND

In 2005, cancer overtook cardiovascular disease as the leading cause of death in individuals under the age of 85 in the United States.[1] In 2008, an estimated 7.6 million cancer deaths are thought to have occurred worldwide, with an incidence rate that has been steadily growing for decades.[2,3] Breast cancer in particular accounts for 22.9% of all cancer occurrences and 14% of cancer related deaths in females, surpassed only by lung cancer as the cause of female cancer-related mortality,[4] with an estimated 255,000 new cases diagnosed in 2017 in the United states alone and responsible for an estimated 595,690 deaths in 2016.[5-7] Advances in early detection methods such as mammography, Positron emission tomography (PET), and magnetic resonance imaging (MRI) have significantly reduced breast cancer mortality, (annual mammography alone has shown to reduce breast cancer mortality by 30% in women 50-69).[8] However, the 5-year survival rate of patients with metastatic breast cancer remains disparately grim at less than 15%, with a median survival time of 18 months.[7,9] Highly metastatic forms of cancer are those whose comprising cells, rather than remaining relatively contained within a primary tumor site, have an inordinate ability to migrate throughout the body and proliferate unchecked. This characteristic makes it very challenging to detect, localize, and ultimately treat the cancer cells effectively. This disparagingly large gap in treatment efficacies by stage demonstrates a clear need for more effective means of treating highly metastatic forms of breast cancer. Intratumoral (IT) administration of chemotherapeutics

is an obvious solution to the question of how to deliver drugs in cancer treatment, and in practice reduces systemic toxicity and increases drug action at local site. While common sense would dictate that this method would be ultimately insufficient for treating metastatic cancers, recent studies have actually shown that IT therapy can serve to generate an immune response against subsequent metastases, and when used prior to surgery on a primary tumor can serve to greatly reduce surgical morbidity or even kill the primary tumor by itself.[10] That being said, some notable shortcomings of IT therapy include non-uniform distribution, rapid drug clearance, and ultimately low penetration rates as a result of the high pressure gradients. Overcoming these problems would require an effective means of both delivering and retaining chemotherapeutics within the tumor site.

1.1 Metastasis and the Metastatic Niche

Due to the competition inherent in the functionality of biological niches, it seems unlikely that the small populations of cells involved in initial metastatic seeding would be capable of producing the quantities of chemokines required for a gradient relevant to that of the primary tumor, indicating the existence of some additional process operating synergistically with those addressed above. In addition to the challenge and cost of investigating the mechanisms of this behavior, previously accepted theories of metastasis based on Steven Paget's original 'seed and soil' hypothesis offered little to support or even justify the existence of such a phenomenon.[11-13]

As the understanding of metastasis has evolved, however, the identification and characterization of pre-metastatic and micrometastatic sites arising from Bernard and Weinberg's dual proclivity model may serve to explain the tropism of cancer cells to these sites.[14-17] Current understanding places strong association between cancer and with the widespread mobilization of inflammatory cells in the blood and hematopoietic cells, however bone marrow derived hematopoietic cells expressing vascular endothelial growth factor receptor 1 (VEGFR1) and the fibronectin receptor VLA4 (integrin α4β1) have been found to localize to pre-metastatic sites prior to seeding by cancer cells.[18,19] The recruitment of these cells are a result of both tumor secreted angiogenic cytokines such as VEGFA and placental growth factor (PIGF, which binds to VEGF1), as well as from S100 inflammatory chemokines and serum amyloid A3 (SAA3) expressed in response to VEGFA, transforming growth factor β (TGFβ), and tumor necrosis factor-α (TNF-α) release from the primary tumor.[20-22] Sites which also exhibit notably higher stromal fibronectin expression which, in conjunction with the accumulated myeloid cells, would serve as effective docking sites for disseminating tumor cells and increase proliferation of cancer cells at these sites.[19,23]

1.2 Current Treatment Methods

Current treatment of breast cancer typically begins with an initial surgical intervention (lumpectomy, partial/full mastectomy, etc.), followed by radiation therapy in conjunction with either hormone therapy, chemotherapy, immunotherapy, or some combination of the three. The immediate challenge of surgical intervention is that not all

forms of breast cancer are easily localized; tumors that do not differ significantly in density or extracellular composition to the surrounding tissue are very difficult to remove completely, and highly metastatic forms are unlikely to be entirely localized to a single tumor.[7]

Hormone therapy, one of the most common means of treating breast cancer, functions through antagonistic interaction with hormone receptors that are overexpressed in cancerous cells, and are understood to play a role in the rapid growth and proliferation of these cells. The three most well-known of these receptors are those for estrogen, progesterone, and HER2, and are the targets for most commercially available hormone therapy drugs[24]

While effective in treating the roughly 85% of breast tumors that exhibit overexpression of at least one of these receptors, the mechanism by which this class of drug functions prevents them from having any therapeutic effect in the 15% of cases where these receptors are not expressed, commonly referred to as triple negative breast cancer.[25] Highly proliferative and aggressive, triple negative breast cancers are typically managed by systemic chemotherapy. In addition to high cytotoxicity and a very narrow therapeutic window, this method of treatment alone is associated with high rates of recurrence, both local and systemic.[26]

As possibly the most well-known method of treatment, chemotherapy through the systemic introduction of cytotoxic agents has long been proven to actively hinder growth and proliferation of cell populations in advanced breast cancer.[27] Anthracyclines, a large class of chemotherapeutic drugs first developed in the early 1960s, remain among the

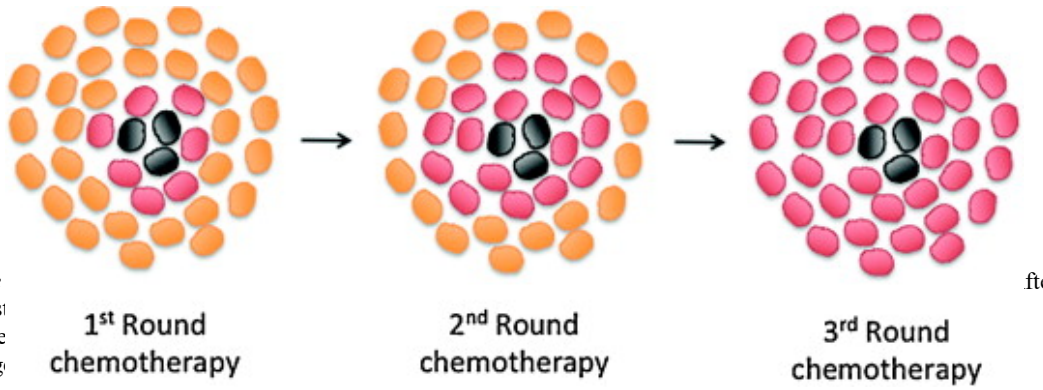
most effective anticancer drugs ever developed (e.g. fluorouracil, doxorubicin, cyclophosphamide).[28] Despite half a century of rapid progress in the field of oncology, one of the first anthracycline drugs ever developed, doxorubicin (DOX, sold under the market name Adriamycin), remains on the World Health Organization's list of essential medicines and recommended for systemic use in metastatic breast cancer; with demand such that as recently as 2014 the United States experienced a national shortage.[29,30]

DOX functions by inducing apoptosis in cells via two distinct mechanisms: (1) Intercalation into DNA, which inhibits the activity of topoisomerase-II, an enzyme which is responsible for unzipping the DNA helix. In doing so, all of the cellular processes dependent on the functionality of topoisomerase-II are subsequently arrested, such as the replication, repair, and transcription of DNA. Intercalation can also result in histone eviction from transcriptionally active chromatin, further deregulating the DNA damage response in DOX exposed cells. (2) Cytosolic conditions equilibrate the oxidation of Doxorubicin to semiquinone in a reversible reaction that serves to produce reactive oxygen species capable of significantly damaging cellular DNA.[31,32]

Occurring simultaneously within a cell, these mechanism cause DOX and other similarly functioning anthracyclines to exhibit potent apoptosis-inducing qualities. However, a number of serious limitations still exist that can severely hinder the effectiveness of clinical treatments dependent on the use of these agents. Depending on the drug, these limitations can include poor retention, non-specific distribution, toxicity, and the development of resistance in cancer cells.[34-36] In fact, the cytotoxic characteristic of these agents that makes them so effective in mitigating the proliferation and growth of

cancerous cells is the very reason why cytotoxic chemotherapeutics alone will never prove to be the oncological panacea. This is due to the fact that cytotoxic agents currently available have no mechanism for selectivity; they affect cancerous and healthy cells indiscriminately. The severe side effects of chemotherapy are a result of the drugs functioning properly; the cell killing effects of the chemo drugs being active only on cancerous cells as well as the healthy cells in bone marrow, gastrointestinal epithelia, hair follicles, and cardiac musculature.[37]

The damage on healthy cells caused by the action of these drugs results in the need for convalescence time between treatments, to allow for recovery of the population of these affected healthy cell types. Anthracycline-based regiments have objective response rates of 50-80%, with median response duration and survival times lasting from 10-18 months and 18-26 months, respectively.[9] The contrast between the purported efficacy of these drugs and the clinical data is likely indicative of the use of suboptimal doses and longer than ideal convalescence times between treatments to prevent acute/chronic toxicities and minimize damage to non-cancerous tissues.[38] Due to the heterogeneous populations of cancer cells in tumors, especially fast growing tumors, this method of treatment fosters the rapid transition from a primarily drug sensitive population to an



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entirely resistant population (Fig. 1). In many cases, the mechanism of the resistance acquired causes diminished efficacy in a range of drugs, often requiring the use of multiple chemotherapeutic agents with very different mechanisms of action over the course of treatment.[39]

1.3 Multidrug Resistance

There are two main pathways by which a population of cancer cells can become multidrug resistant (MDR), one being pump dependent and the other being non-pump dependent.[40] Non-pump dependent MDR is characterized by an upregulation of proteins that inhibit the apoptotic pathway; Bcl-2 being the most commonly observed in this form of drug resistance.[41] Since most common chemotherapeutic drugs such as doxorubicin or paclitaxel function within the cell by triggering apoptosis, non-pump dependent MDR can retard or even eliminate the efficacy of drugs while still facilitating an environment promoting the development of pump-dependent resistance.[42]

Pump dependent MDR is characterized by an overexpression of P-glycoprotein 1, also known as multidrug resistance associated protein 1 (MDR1), a transmembrane drug efflux pump that functions to rapidly expel therapeutic agents from the cytosol into the extracellular domain before they can reach their site of action. This overexpression of MDR1 results in both a diminished efficacy of the drug and an increase in extracellular toxicity,[42] and has been found to be a major contributor to chemotherapy failure in different MDR-overexpressing cancer types.[43-45] This mechanism of drug resistance is particularly effective since, once developed, it can remove a wide range of drugs indiscriminant of their mechanism of action. While the use of drugs functioning via an

alternative mechanism of action has shown to be an effective method of mitigating the action of non-pump dependent resistance, overcoming pump dependent MDR has shown to require more complex methods of treatment, involving either drugs with a novel mechanism of inhibiting activity of efflux pumps, or some means of preventing expression of the genes coding for them.[46] In order to most effectively overcome drug resistance in cancer populations to allow for successful long-term treatment with cytotoxic agents, simultaneous inhibition of both mechanisms must occur.[47,48] Lastly, co-treatment of cancer cells exhibiting MDR with siRNA-mediated gene silencing and chemotherapeutic drugs, administered separately, has shown to improve the overall safety and efficacy of treatment, however it is very likely that co-delivery of resistance-inhibiting siRNA with chemotherapeutic drugs would be more efficient in treating cancer cell populations exhibiting MDR.[36, 49]

CHAPTER 2

GENE THERAPY IN CANCER TREATMENT

Gene therapy has shown great promise in treating gene-related disorders. Since the first successful clinical trial in 1990 treating Adenosine Deaminase Deficiency (ADA),[50] research in gene therapy has exploded from rare monogenic diseases to include potential applications in the treatment of complex conditions like cancer.[51] Typical gene therapy approaches can be divided into two major categories based on action: function enhancing (such as through introduction of pDNA), and function inhibiting (such as gene silencing through RNAi). Gene silencing through RNA interference (RNAi), can effectively inhibit the expression of nearly any gene with high efficiency and specificity, and in doing so stop production of target proteins regardless of their function or structure.

2.1 Function Interference

The RNAi pathway is initiated by the presence of long double stranded RNA (dsRNA) (>200 base pairs) in the cytosol, which activates the enzymatic complex Dicer. The dsRNA is then processed and cleaved by Dicer into twenty-two double stranded fragments (20-25 base pairs) of small interfering RNA (siRNA) with a two nucleotide overhang on the 3' ends of either strand. The dicer products then assemble with complexes containing endoribonuclease, which makes up the RNA-induced silencing complex (RISC), at which point the RNA strands separate, with the sense strand remaining in the cytosol to be degraded and the antisense strand remaining bound RISC,

activating the complex. Once activated, the RISC then identifies, binds, and cleaves any messenger RNA (mRNA) complimentary to the antisense strand bound to the RISC, preventing translation and selectively silencing gene expression.[52,53]

Long dsRNA can be delivered to initiate the RNAi pathway, however the use of long dsRNA for gene therapy has been shown to present a number of delivery challenges. These challenges include transport through the plasma membrane, protection from serum nucleases, and preventing immunogenesis, all of which would make actual implementation in a therapeutic application impractical.[54] Synthetic siRNA, however, bypasses several of the challenges of long dsRNA due to its reduced size, such as easier encapsulation in small delivery vehicles, and reduced immunogenicity.[55,56] It is likely due to these reasons that siRNA is most common structure used in RNAi-based therapeutics.[53]

In most studies attempting to use gene therapy in the treatment of cancer, target genes involved in the apoptotic or proliferative pathways are exploited to improve efficacy of adjuvant therapies.[57] These have included function enhancing approaches delivering pDNA coding for proapoptotic genes such as TNF-α[58] and p53,[59] as well as function inhibiting approaches, delivering siRNA targeting antiapoptotic or resistance-mediating genes such as Bcl-2[60] and MDR proteins.[61] In particular, studies examining siRNA-mediated silencing of MDR proteins have shown some success in re-sensitizing cells to chemotherapeutics and improving treatment with anticancer drugs.[62-65] That being said, few products have made it as far as clinical trials this far,[66] often due to issues challenges of siRNA delivery.

2.2 Barriers to Treatment

Gene therapy utilizing the RNAi pathway has enormous potential for clinical application; however, several major physiological barriers stand in the way of an effective siRNA-based therapeutic reaching the market anytime soon. In order to have any effect on gene expression, siRNA molecules must be delivered directly to the cytosol of target cells. Injection of naked DNA/RNA has been empirically shown to be fairly ineffective as a means of drug delivery within this field; the large size, negatively charge phosphate groups, and relative hydrophilicity all present immediate challenges individually capable of preventing nucleic acids from diffusing through cell membranes under normal conditions (e.g. passive diffusion), meaning that a delivery system that can selectively and efficiently deliver a gene to target cells is needed for successful gene therapy to occur.

In addition to mediating endocytosis, an effective delivery system must be able to overcome (among others) several major barriers to successful gene silencing *in vivo*. These include: (i) a means of specificity, both in biodistribution as well as cellular uptake; (ii) protection against degradation by serum ribonucleases; (iii) preventing and delaying particle/siRNA clearance through the renal and reticuloendothelial systems; (iv) a mechanism to avoid or at the very least minimize interaction with serum proteins and non-target cells (it is in this regard, primarily, that bPEI falls short in satisfying all the requirements of an effective gene carrier); (v) minimization of complex immunogenicity and cytotoxicity in systemic circulation, and finally, (vi) to facilitate delivery into the cytosol of cells within the target population.[53]

Another major barrier to the delivery of siRNA *in vivo* is the need for particle protection in systemic circulation – be it from ribonucleases, serum proteins, non-target cell interactions, elution, or targeting by the immune system. Ribonucleases present in the blood will rapidly degrade free nucleic acids unless otherwise shielded from them. While this can be overcome through sheer volume of systemically introduced RNA or DNA, this process is far too inefficient for clinical application and can initiate an immune response, therefore a means of protecting the siRNA while in circulation is necessary.[67] Additionally, any non-native particles such as free nucleic acids also stand the risk of aggregation with serum proteins and non-target cells. Loading and protection from degradation of siRNA could be accomplished relatively easily via covalent binding or encapsulation within the particle, however the former of these methods would be unsuitable for siRNA mediated gene silencing due to its mechanism of action – free siRNA must be present within the cytosol of target cells to initiate assembly and activation of RISC. Therein lies one of the more complex aspects of delivering nucleic acid as opposed to traditional therapeutics – the siRNA must be bound to a vector such that it remains associated and protected by the particle in circulation, while readily dissociating from the complex once in the cytosol.[68]

One potential method of binding siRNA to a particle that would allow this to occur is through electrostatic interaction, possible due to the strong negative charge associated with nucleic acids as a product of the contained phosphate groups. Complexation via electrostatic interaction with positively charged compounds would have the potential to facilitate both an appropriate binding strength and a means of spontaneous complexation.

While degradation by serum ribonucleases is a fairly simple challenge to overcome through the use of an appropriate drug delivery vehicle, the avoidance of protein binding has proven to require a more complex solution. With regards to opsonins of the adaptive immune system (e.g. antibodies) and cells of the innate immune system, overcoming this barrier has proved a challenge within the field of drug delivery for decades, with limited success.[69] One of the most common methods of mediating protein binding (and subsequently opsonization) of nanoparticles is through the conjugation of a shielding group to the outer surface of the polymer. Some examples of shielding groups that have been heavily tested include polyacrylamide, poly(vinyl alcohol), and poly(N-vinyl-2-pyrrolidone), however more recent studies have shown a clear preference to utilize polyethylene glycol (PEG), and PEG-containing copolymers.[70-72] As evident by the examples listed, these shielding groups tend to be non-ionic surfactants with long hydrophilic polymer chains, which function to mediate protein binding through the formation of a hydration shell around the particle.[70] In doing so, PEGylation can dramatically increase retention time in systemic circulation by effectively 'hiding' particles from serum proteins that would otherwise bind to them and target them for RES uptake.[73,74] This has shown to be very effective, largely due to the fact that hydrophobic and electrostatic interactions are the primary forces involved in protein adsorption.[75] Despite the advantages associate with the use of cloaking polymers such as PEG, its incorporation onto nanoparticles for gene therapy presents several drawbacks as well. The effectiveness of PEG in surface charge shielding and preventing protein adsorption is also likely the cause of reduced complexation with nucleic acids shown in particles that

have been PEGylated, as well as diminished endocytosis and transfection efficiency.[39,76] More recent studies have also begun to indicate that PEG may in fact be more immunogenic than previously thought, which would challenge the candidacy of the polymer for use in long-term treatments.[77]

As mentioned, many barriers exist to siRNA delivery that do not require attention in the delivery of pDNA. Despite all their similarities, pDNA and siRNA behave very differently due to some important differences. Stability is a major concern with RNA, which are vulnerable not only to base-catalyzed hydrolysis by their 2'-OH groups and the ribonuclease abundant in biological environments, but as siRNA are also susceptible to auto-hydrolysis at the 3' overhangs necessary for optimal function.[78] Another major difference that could have serious implications in behavior is size. The radically smaller size of siRNA, with a typical length of 18-25 bp, in comparison to pDNA, which could range anywhere between 1 and 200 kb,[79] could result in very different behaviors in both drug delivery vehicles (DDVs) and a physiological environment– especially because their length is directly linked to overall charge. These factors must be kept in consideration when attempting to translate a vehicle previously used in pDNA delivery to that of siRNA

2.3 Nanoparticles in Drug Delivery

To first focus on specificity of delivery, while in systemic circulation an effective drug delivery vehicle must facilitate deposition within a specific area of activity- in the case of cancer this almost always means particle accumulation within a tumor or tumors. In this regard, the utilization of nanoparticles for drug delivery has a marked advantage

over other methods due to the utilization of the enhanced permeability and retention (EPR) effect as a means of passive targeting. The EPR effect is an intrinsic characteristic of colloidal particles in circulation, in which they exhibit systemic circulation times significantly higher than would otherwise be expected, and therefore an increased bioavailability and ability to permeate specific bodily tissues such as tumors.[80] This effect is observed in particles with a diameter between approximately 10-100 nanometers, a size range at which the particles are too large for rapid elution through glomerular filtration by the renal system, while at the same time remaining too small for efficient identification and opsonization by the reticuloendothelial system (RET).[81,82] The growth process and subsequent structure of tumors serves to create a number of important physiological characteristics that ultimately compound the impact of the EPR effect when used in oncological applications. Rapid angiogenesis, a hallmark of advanced and aggressive cancers, occurs during the both initial formation and tumor growth, as the high nutritional demands of the rapidly proliferating cells produce an environment that too hypoxic and nutrient-deficient to support themselves via diffusion at more than 1-2 mm removed from a blood supply.[83-85] They respond to this deficiency by secreting a range of angiogenic growth factors, recruiting nearby vasculature to produce a huge network that due to rapid formation and chaotic cell growth is inefficient and disorganized.[84] The poorly developed vascular junctions within this system are highly discontinuous.[85] These openings allow for the passage of particles that are too large to pass through any smaller endothelial junctions such as the fenestrations of renal endothelium, much less the minute gap junctions of normal healthy vascular endothelium. This is significant in that the

circulating nanoparticles will be capable of not only highly permeating the tissue of tumors, but also doing so in a significantly higher degree relative to healthy tissues – thereby introducing a favorable degree of specificity with regards to particle biodistribution.[86] Another unique characteristic of tumors is the incredibly poor hemodynamics, which arise partly from their poorly organized blood supply and partly from the complete lack of lymphatic drainage systems found in tumors. In the case of nanomedicine and EPR effect, this hemodynamic property serves to further encourage the retention of particulates within the previously specified size range.[88]

2.4 Current Strategies

The use of nanoparticles for application in clinical medicine is a practice that has been heavily researched since the early 1970's, and has led to the development of a plethora of particle designs, varying wildly by size, composition, structure and function. Of that selection, there are two overarching classes of nanoparticle primarily considered as potential vectors for gene therapy: viral and non-viral vectors. Both of these carrier types have several subclasses with their own strengths and weaknesses that must be considered and weighed in relation to the application.

Viral vectors are created through the use of a functional virus, in which the genes encoding a therapeutic protein have been inserted into a viral capsule so as to express the therapeutic RNA endogenously, while removing the genes responsible for viral activity that would be detrimental to the patient, such as viral DNA synthesis, and the production of reverse transcriptase/integrase.[89] Academic interest in viral vectors has waned over the last decade however, likely due to the severe potential drawbacks and the little-

understood mechanisms behind them. Once a heavily researched topic, viral vectors have a number of advantages in comparison to non-viral alternatives including the potential for long-term expression of the target gene from only a single injection.[90] Additionally, this biologically-derived approach builds upon thousands of years of evolutionary progress to the purpose of delivering genetic material to host cells, the result being a vector that is incredibly effective at transfecting even non-dividing cells such as those of the central nervous system.[91]

While highly infective, many viral capsules are seriously limited by size, impacting their ability to deliver more complex RNA sequences.[92] Adenoviral vectors, which have a fairly large capacity relative to other viral vectors and a linear double-stranded DNA genome, have proven to be applicable and actually quite effective in RNAi delivery.[89] Unfortunately, Adenoviral vectors also have the potential to elicit a strong immune response and liver toxicity.[93] Adeno-associated viruses (AAV) have also been explored as vectors for gene therapy due to the fact that unlike the larger adenoviral vectors, AAV's are non-pathogenic to humans, however they are also significantly smaller (~5.2 kB) and like adenoviral vectors are only transiently expressed.[94,95] As long-lasting vectors capable of integration into a host's genome, vectors produced from Retroviral and lentiviral (a subclass of retrovirus) sources have shown huge potential in the treatment of genetic disorders and even in suppression of diseases such as HIV-1, however like adenoviral vectors, serious concerns exist regarding the safety of these vectors.[95,96] In summary, viral vectors have been shown to have huge potential in gene therapy applications, however a number of limitations present in all viral vectors regardless of subclass must

first be addressed, including limited loading capacity, immunogenicity, and risk of wild-type virion regeneration.[97-99]

Non-viral vectors for gene therapy are artificially produced carriers designed to assemble with siRNA to form complexes capable of delivering their complex genetic material to the cytosol of target cells. While much safer than their viral alternatives, non-viral vectors come with their own drawbacks.[68] Non-viral vectors have an effect that is inherently transient, and unlike viral vectors, they must address challenges such as mediating particle endocytosis and endosomal escape in the design of the particle. Subclasses of non-viral vectors include, among others, liposomes, lipoplexes, and cationic polymers.

Liposomes are highly ordered nanoparticles that are composed of a lipid bilayer envelope which encapsulates an internal aqueous phase. In many ways liposomes are biomimetic structures, very closely resembling the phospholipid bilayer of the plasma membrane in cells. Liposomal nanoparticles have long been used for applications in drug delivery, having proven to be especially effective in delivering hydrophilic drugs via encapsulation within its aqueous core. Other notable advantages of using liposomes for drug delivery are its low cytotoxicity, ease of surface modification, and potential for self-assembly.[100] Unlike other non-viral vectors for gene therapy, liposomes can be loaded with siRNA just as easily as with any hydrophilic drug – in many cases by simply mixing solutions of the two components – loaded safely within the cell simply by encapsulation.[100] Some drawbacks of liposome use for gene therapy include poor transfection efficiency and the lack of any means of mediating endocytosis or endosomal

escape, as well as a high risk of opsonization. However, these can be overcome via surface modification with other bioactive compounds capable of addressing these challenges.[101]

Similar in composition to Liposomes, lipoplexes are formed when cationic lipids electrostatically complex with nucleic acids to produce a stable molecule capable of binding and protecting siRNA in systemic circulation.[102] The use of cationic lipids provides several advantages over uncharged liposomes, the foremost being its ability to spontaneously associate with siRNA and the lipid bilayer of cells. This results in much higher cell internalization, both by endocytosis and by disruption of the cell's plasma membrane (PM). The presence of a strong positive surface charge on these particles also has the potential to increase cytotoxicity, as well as phagocytosis/capture via the RES, which can drastically reduce residence time.[103,104]

Cationic polymers, like cationic lipids, also spontaneously complex with siRNA due to ionic interactions between the cationic groups within the polymer and the phosphate groups of the siRNA to form a stable particle, with many similar properties to lipoplexes. Excessively high surface charges in these vector designs can result in high cytotoxicity and even spontaneous aggregation with abundant negatively charged blood proteins such as albumen and fibrinogen, which typically results in the rapid elimination from systemic circulation.[81] While charge shielding could be accomplished via conjugation with stealth polymers, a major advantage of using cationic compounds to bind and deliver anionic nucleic acids is that the final surface charge of the complex is a function of the ratio between the protonated amine groups in the carrier's structure and the negatively charged

phosphate groups of the bound nucleotides. This ratio is commonly described by the N/P ratio, and can be optimized based on the specific vector being examined. This system allows for the optimization of particle loading in terms of balancing toxicity and transfection efficiency. With exceptions to polymers conjugated to targeting moieties, most cationic polyplexes facilitate intracellular uptake by nonspecific endocytosis through interaction with heparin sulfate proteoglycans (HSPGs) present in the extracellular matrix.[105] For this reason, polyplexes that maintain a slightly positive surface charge usually show improved stability and interaction with cell membranes,[106,107] however this charge can also produce certain undesirable effects when used *in vivo* such as serum protein induced aggregation and excessive interaction with plasma membranes.[108]

One well-known example of a cationic polymer used in polyplex formation is polyethylenimine (PEI). PEI is considered to be the 'gold standard' of non-viral gene delivery due to its high cellular uptake and endosomolytic activity, both essential aspects of transfection.[109,110] While low molecular weight (MW) PEI variations have been investigated and found to have a somewhat lower cytotoxicity, high MW (25 kDa) PEI is much more commonly seen in studies investigating cationic polyplexes for gene therapy, likely due to its significantly higher transfection efficiency.[39,111-113] This is because high MW PEI is incredibly effective in mediating both endocytosis and endosomal escape, two of the most challenging barriers to overcome with non-viral vectors. The branched version of PEI in particular (bPEI) has been shown to have highest efficiency and cytotoxicity – a result of the presence of primary amines throughout the branched

variation and their affinity for protonation at physiological pH compared to the secondary amines comprising linear PEI (lPEI) (for the remainder of this paper, it can be assumed that PEI is in reference to high MW bPEI unless otherwise specified).[114,115] Other popular cationic vectors for siRNA delivery include polyamidoamine (PANAM) dendrimers, polylysine (PLL), and chitosan, however like PEI these polymers have not seen much success *in vivo* due to similar issues with efficiency and toxicity in biological conditions.[106,116,117]

2.5 Endocytosis and the Proton Sponge Effect

While PEI has been reported to enter the cell via a combination of mechanisms partially dependent on particle size, having been documented entering cells through clathrin and caveolin-mediated endocytosis as well as macropinocytosis, recent studies have shown that these mechanisms are not solely responsible for all PEI endocytosis.[118-120] One proposed hypothesis to account for this finding, as well as the synergistic effect of cationic charge on siRNA delivery is that ionic interactions between the cationic polymer (such as PEI) and the PM result in the transient disruption of the PM to create nanoscale holes capable of permitting particle transport into the cell.[121] Interestingly enough, the same interaction between cationic polymers and the outermost bilayer has been proposed by several research groups as the cause of the cytotoxicity seen in cationic polymers.[122,123] While particles entering the cell through this proposed mechanism would be safe from endosomal degradation, the high transfection efficiency of PEI compared to other cationic polymers indicates the action of another mechanism at work – endosomal escape.

Endosomal escape is a critical function of an effective gene carrier, as it can dramatically improve transfection.[124] Failure for endosomal escape to occur would otherwise result in complex degradation from the acidic environment as well as the proteases and peroxidases that accumulate as the early endosome reaches maturation.[125] The most commonly accepted mechanism that has been proposed to explain PEI's apparent ability to escape the early endosome is known as the "proton sponge" effect, hypothesized to occur with other cationic polymers such as PAMAM and chitosan, and utilize the buffering capacity of tertiary and secondary amine groups to inhibit endosomal acidification. The theory proposes that as the pH begins to drop in the early endosome, protonation of amine groups in the polymer that exhibit pK^a values between neutral and lysosomic pH inhibits further acidification of the endosome. While reports from Benjaminsen et al. have indicated that this is not in fact the case, and that the endosome eventually reaches a normal pH not below 5.5,[126,127] this is not to say that his results invalidate the theory – in fact Benjaminsen and Richard et al. both argue that these results simply indicate that while functioning as a 'proton sponge,' the V-ATPase pump responsible for stabilizing the pH of the endosome is capable of overcoming this effect.[126,127] In any case, the buffering capacity of the PEI results in the influx of protons to the endosome by the V-ATPase pumps in the process of stabilizing endosomal pH. The presence of both free protons and the protonated primary and secondary amine groups within the PEI result in the formation of an electrochemical gradient responsible for the endosomal transport of large chloride ions into the endosome, in turn creating an osmotic gradient responsible for the influx of water into the endosome. The influx of

these protons, counter-ions, and water result in the swelling and ultimately rupturing of the endosome, releasing its contents into the cytosol.[128,129] Once taken up by a target cell and exposed to the acidic conditions, the siRNA should be able to disassociate from the complex and diffuse throughout the cytosol.[130]

2.6 Poly(ethylenimine) and Strategies for Improvement

While touted as the gold standard of non-viral vectors in gene delivery, there still exists a significant gap between PEI's impressive efficiency in non-serum conditions and its relative ineffectiveness in serum conditions.[131,132] Most likely this is a result of particle stability being less than ideal, the polymer strands comprising the complex readily aggregating to the abundant anionic serum proteins or erythrocytes in the blood.[133] This explanation for the performance of PEI seems reasonable when considering the ionic interactions of the nucleic acids and the cationic polymer are the only forces holding the complex together.[68] PEI's poor serum performance being a result of complex instability is further supported by the drop in transfection efficiency and aggregation observed in lyophilized samples as well as samples diffused out of hydrogel-based scaffolds.[134-136] One promising method of improving cationic polyplex stability that has been investigated is grafting with hydrophobic polymers. In addition to improving colloidal stability, the use of amphiphilic block copolymers for complexation with nucleic acids has been shown to increase transfection efficiency and decrease cytotoxicity.[131,137,138] Specifically, vectors that assemble into a micelle formation through incorporation of hydrophobic polymers also have shown to reduce charge density and improve the particle's capacity to facilitate endosomal escape.[139] In the specific case of bPEI, one of the most effective modifications

of the polymer to date has been accomplished by conjugation with low MW hydrophobic groups.[140] Micelle formation is primarily driven by two highly cooperative forces: hydrophobic interactions between the hydrophobic regions of the amphiphilic polymer, and electrostatic attraction between the negatively charged nucleic acids and the cationic regions of the polymer.[141, 142] Micelles will form spontaneously at or above the critical micellar concentration (CMC), and have shown to contribute heavily to particle stability.[143]

Having established the need for effective drug delivery systems for the application of both chemotherapeutics and nucleic acids, as well as the advantages of co-delivery over just concurrent treatment, the need for a multifunctional vector capable of specific and combinatorial delivery of these therapeutic agents is quite apparent.[144] While this particle would necessarily be used in adjuvant treatments, the successful implementation of such a complex could represent a sorely needed advancement on current treatments.

CHAPTER 3

RESEARCH AIMS

3.1 Objectives

While many advances in oncology have improved patient outlook and overall mortality, the development of multidrug resistance remains a critical obstacle in the development of treatments in breast cancer. The goal of this research is to develop a dual-functional nanotherapeutic for the mitigation of multidrug resistance in metastatic breast cancer. This study is designed around the hypothesis that combinatorial therapy with doxorubicin and siRNA silencing P-glycoprotein will serve to concurrently re-sensitize drug resistant cells to critical chemotherapeutics while delivering said drug to the newly sensitized cells. This study will focus on *in vitro* analysis of both doxorubicin and siMDR1 delivery to cells as mediated by PgP.

3.2 Vehicle Design

The cationic micellar copolymer Poly(lactide-co-glycolide)-graft-poly(ethylenimine) (PgP) is a novel design (figure 2) synthesized by conjugating the cationic polymer PEI to the hydrophobic block copolymer PLGA, creating a structure that should retain the characteristics of PEI that made it such a promising vehicle for gene therapy – namely its ability to electrostatically bind and intracellularly delivery nucleic acid, as well as its buffering capacity within early endosomes. A major barrier of PEI to use in therapeutic applications is its relative inability to function in serum conditions due

to issues of stability and interaction the charged particles present in high protein conditions. By conjugating the polymer to PLGA, the produced particle should exhibit improved stability due to hydrophobic interactions and the spontaneously arising micellar structure of the particle at concentrations above the critical micellar concentration (CMC). The hydrophobic PLGA core should be capable of loading hydrophobic drugs while providing stability to the particle, with the PEI in the corona of the micelle being able to electrostatically bind nucleic acids such as pDNA or siRNA due to the presence of cationic amine groups. The complexed particle size has been characterized at a diameter of approximately 120 nm, within optimum range for exploitation of the EPR effect – being too large for rapid expulsion by glomerular filtration but too small for targeting by opsonization and elimination by the reticuloendothelial system – while passively targeting and accumulating in tumorous tissue as a result of the poor hemodynamics and endothelial disfunction arising from their rapid and disorganized growth. Once accumulated, it can be endocytized, facilitating endosomal escape into the cytosol via the

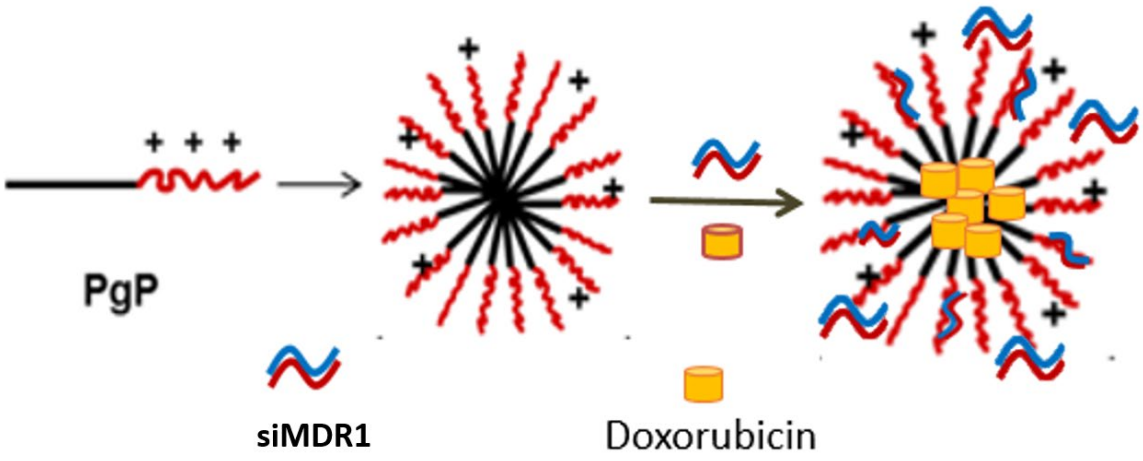

Figure 2. PgP Micelle Design. The micellar structure of PgP, where black lines represent the PLGA blocks and red lines represent PEI chains within the PgP polymer.

proton sponge effect – an inherent characteristic arising from the buffering capacity of secondary amine groups within the PEI branches. Once in the cytosol, their nucleic acid load can begin to function, and the action of any loaded anthracycline-class chemotherapeutics will be drastically be improved by delivery to the site of action.

3.3 Study Outline

In order to determine the ability of PgP to function as designed, the suitability of PgP as a delivery vehicle for both siRNA and hydrophobic chemotherapeutics must be examined. The stability of PgP/siRNA will be assessed, as well as its ability to function *in vitro* in simulated physiological conditions (i.e. in serum). Initially, this will be determined by testing the ability of PgP to transfect MDA-MB-435 ADR and wild type cells with pGFP, as well as the cytotoxicity of the polymer in both cell types. Should the initial results show PgP to be an effective vector for pDNA delivery with minimal toxicity in this cell line, stability can be determined by heparin competition assay to determine the stability of the PgP/siRNA micelles in competition with other charged particles, as well as the stability of siRNA-bound complexes relative to the pGFP-bound complexes, which have already been shown to be effective in transfection of other cell lines. This will be followed by *in vitro* analysis of both cytotoxicity and its ability to successfully silence the MDR1 gene, on both the mRNA and protein level, in MDA-MB-435 ADR cells when complexed with siMDR1. Doxorubicin loading in PgP will also be assessed, through photospectrometry to determine loading efficiency, and then cytotoxicity by MTT assay of MDA-MB-435 ADR cells treated at varying concentrations

to determine the optimum loading concentration. Finally, the practical efficacy of co-delivering doxorubicin with siMDR1 will be assessed by cytotoxicity studies of both co-delivered siMR1 and doxorubicin in the form of DOX/PgP/siMDR1 complexes. Future studies can include *in vivo* antitumor, biodistribution, and toxicity studies in athymic nude mice using induced MDA-MB-435 ADR tumors and intratumoral treatment with the fully loaded and complexed particle.

CHAPTER 4

MATERIALS & METHODS

4.1 Materials

Poly(lactide-co-glycolide) (PLGA, ~4 kDa 50:50) was purchased from Durect Corporation (Pelham, AL), with a carboxylic end group. Branched poly(ethylenimine) (PEI, 25 kDa), as well as Thiazolyl Blue Tetrazolium Bromide, and RPMI 1640 was purchased from Sigma-Aldrich (St. Louis, MO). The wild type (WT) and Doxorubicin-resistant (ADR) MDA-MB-435 cells were provided by Dr. Hassan Uludag's group at the University of Alberta (Alberta, Canada). FBS was acquired from Atlanta Biologicals (Norcross, GA). 200 nM L-Glutamine, as well as 0.25% trypsin/2.21 mM EDTA in Hank's Balanced Salt Solution were purchased from Mediatech Inc. (Manassas, VA). Doxorubicin (DOX) was purchased from LC Laboratories (Woburn, MA). Doxorubicin Hydrochloride (DOX-HCl) was purchased from MP Biomedicals LLC (Solon, OH). Formaldehyde Loading Dye used in gel electrophoresis was purchased from Ambion Inc. (Waltham, MA). Molecular weight ladders (1 kb and 100 bp DNA Ladders), and Penicillin/Streptomycin (10,000 units/mL Penicillin, 10 mg/mL) were purchased from Gibco (Grand Island, NY). Plasmid DNA encoding the Monster Green Fluorescent Protein (pGFP) was purchased from Promega (Madison, WI). The siRNA targeting MDR1 (ABCB1 or P-glycoprotein, NCBI reference sequence: NM_000927.4), ID 4123 and Negative Control 1 siRNA (siNT) were obtained from Ambion Inc. (Waltham, MA). The TURBO DNA-Free Kit, HALT Protease & Phosphatase Inhibitor Cocktail, Pierce

BCA Protein Assay kit, SuperSignal West Pico Chemiluminescent Substrate, High-Capacity cDNA Reverse Transcription kits, 6x DNA Loading Dye, Albumin Standards, and the Lipofectamine 3000 used as a positive control in transfection, were all obtained from Thermo Fisher Scientific (Waltham, MA). Mouse monoclonal anti-β-actin antibody (1:1,000) and mouse monoclonal anti-MDR1 antibody (1: 1,000) was purchased from Santa Cruz Biotechnology (Dallas, TX). Goat anti-mouse HRP-conjugated antibody was purchased from Southern Biotechnology (Birmingham, AL). 4x Laemmli Sample Buffer, Immuno-Blot PVDF membranes, Molecular Biology Agarose, and Precision Plus Protein Kaleidoscope Protein Standards used in western blotting were purchased from Bio-Rad Laboratories (Hercules, CA). RNeasy Mini Plus RNA isolation kit, Quantitect SYBR Green PCR kit, and Maxi Plasmid DNA Purification kits were purchased from Qiagen (Valencia, CA). Primers for RT-PCR were custom designed and purchased from Integrated DNA Technologies (Skokie, IL), with sequences as given: Forward Human GAPDH 5'- CAC CCA CTC CTC CAC CTT TG -3' Reverse Human GAPDH 5'- CCA CCA CCC TGT TGC TGT AG -3' Forward Human MDR1 5'- TCG CCT GGA TTC CCT CCT C -3' Reverse Human MDR1 5'- AGG TCA GCA GAG CCA AGG AG -3'

4.2 Synthesis of Poly(lactide-co-glycolide)-g-poly(ethylenimine)

The Cationic and amphiphilic copolymer poly(lactide-co-glycolide)-g-poly(ethylenimine) (PgP) was synthesized according to methods previously described by Gwak et al, in which 4 kDa Poly(lactide-co-glycolide) (PLGA, 50:50) containing a carboxylic end group was conjugated to the primary amine group of 25 kDa

polyethylenimine, branched, (bPEI) by ester bonding. Once synthesized, the produced PgP was then purified through dialysis against deionized water using a membrane filter with a molecular weight cut off (MWCO) of 50 kDa. To remove any unreacted PLGA precipitate present the PgP was then centrifuged for 10 minutes at 5,000 rpm. The purified PgP was then lyophilized and stored at -20 °C.

Structure and molecular weight of PgP was confirmed by ^{1}H-NMR and gel permeation chromatography (GPC), respectively, as previously described.[132]

4.3 Plasmid Amplification and Purification

The plasmid encoding Monster Green Fluorescent Protein (pGFP) was obtained from *Escherichia coli* DH5α Transformed and amplified in Lysogeny broth on a shaker plate at 250 rpm for 12 hours at 37 °C. The Endofree Maxi Plasmid Purification Kit was used to isolate and purify the plasmid according to the manufacturer's protocol. Purity and concentration of pGFP was determined via spectrophotometry using the Biotek Synergy HT plate reader.

4.4 Cell Culture

The human cell line MDA-MB-435 (both WT and ADR) were cultured in RPMI 1640 cell medium supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (p/s), and 1% L-Glutamine at 37 °C and 5% CO_2. Drug resistance in MDA-MB-435 ADR cells was maintained through weekly treatments of 0.2 μg/mL DOX-HCl.

4.5 Transfection Efficiency and Cytotoxicity of PgP/pGFP Complexes

4.5.1 Transfection Efficiency of PgP/pGFP polyplexes

Transfection efficiency of PgP/pGFP polyplexes was determined by transfection of MDA-MB-435 ADR and WT cells with PgP/pGFP complexes, followed by analysis with fluorescent imaging and flow cytometry.

MDA-MB-435 cells (1.2 x10^5 cells/well) were seeded in 12-well plates containing 1 mL of complete media and cultured for 24 hours. Media was then aspirated and replaced with RPMI 1640 supplemented with 10% FBS and 1% L-Glutamine, and PgP/pGFP complexes added. PgP/pGFP complexes were prepared immediately prior to transfection, and were formed at N/P ratios of 30:1, 45:1, and 60:1 in sterile deionized water and incubated for 30 minutes at 37 °C. Controls used in this experiment included untreated cells, cells treated with naked pGFP, and cells treated with pGFP complexed with PEI at a N/P ratio of 7:1. Cells were then incubated for 24 hours, at which point all wells were washed three times with media and were left to incubate an additional 24 hours.

At 48-hours post-transfection, GFP-expressing cells were imaged using an inverted fluorescent microscope. After imaging, cells were washed with PBS and incubated for 10 minutes in 250 μL of 0.25% trypsin. 500 μL of media was then added to each well, and samples were briefly triturated before being removed to Eppendorf tubes for analysis by flow cytometry. Results were gated by size to eliminate cell fragments and groups of clumped cells, and by fluorescence set as the lowest point at which

untreated cells showed 0% transfection in order to control for low levels of auto fluorescence.

4.5.2 Cytotoxicity of PgP complexed with pGFP

To determine the cytotoxicity of PgP as a vector for pDNA, a cytotoxicity study was performed in parallel experiments to the transfection efficiency study outlined in section 3.8.1. and using the same transfection procedure given above. MDA-MB-435 cells were used for this experiment as well, transfected with PgP/pGFP polyplexes complexed at N/P ratios of 30:1, 45:1, and 60:1 in sterile deionized water, and control groups once again included untreated cells, cells treated with naked pGFP, and cells treated with pGFP complexed with PEI at a N/P ratio of 7:1.

Cell viability was then determined by MTT assay performed according to the following procedure. At 48-hours post-transfection, all wells were rinsed three times with media. Cells were then incubated for 4 hours at 37 °C in 500 μL of serum-free media and 120 μL of 2 mg/mL Thiazolyl Blue Tetrazolium Bromide (TBTB) in PBS. After incubation, cells were rinsed with PBS and 1 mL of DMSO added to all wells. The plate was wrapped in foil and placed on an orbital shaker for 10 minutes to allow for complete dissolution of the formazan crystals formed during their incubation with TBTB. DMSO was removed to a 96-well plate and the absorbance at 570 nm measured in triplicate using a Biotek Synergy HT plate reader. Relative cell viability was calculated as Cell Viability (%) = $(OD_{570\ (sample)}/OD_{570\ (control)})*100$

4.6 Heparin Competition Assays

While the ability of PgP to successfully bind, protect, and deliver pDNA has been previously established,[132] this study was proposed in order to determine the binding strength of PgP to siRNA relative to that of pDNA. 1% and 2% agarose gels were prepared with a 12-well comb and used for pGFP and siMDR1, respectively, both stained with 0.2% ethidium bromide. Polyplexes were prepared at N/P ratios of 30:1, 45:1, and 60:1 with either pGFP or siMDR1, controlling for 0.1 μg of nucleic acid per well. Samples were then incubated for 30 minutes at 37 °C to allow stable and consistent complex formation. Heparin was added to the complexed samples in varying concentrations, measured by weight ratio of heparin to nucleic acid in the sample. After the addition of heparin, samples were incubated for one hour at 37 °C and then loaded into the gels using formaldehyde loading dye or 6x DNA loading dye. Samples were electrophoresed at 80 V for 60 minutes, and visualized with the ChemiDoc-It system using an ethidium bromide filter and UV illumination.

4.7 Knockdown Efficiency and Cytotoxicity of PgP/siMDR1 Complexes

4.7.1 Cytotoxicity of PgP/siMDR1 Complexes

In order to evaluate if PgP/siMDR1 complexes cause toxicity to cells, whether due to the particle, the introduction of siMDR1, or by any other means, a cytotoxicity study was performed. MDA-MB-435 ADR cells (6 x10^4 cells/well) were seeded into 24-well plates containing complete media and cultured for 24 hours prior to transfection. Following the previously outlined transfection procedure, PgP/siMDR1 complexes

formed at N/P ratios of 30:1, 45:1, and 60:1 in sterile nuclease-free water were added to wells with 500 μL of complete media. Control groups for this experiment included untreated cells, and cells treated with Lipofectamine 3000 carrying siMDR1. Dose was controlled for treatment groups by siMDR1 amount: 1 μg/well. One deviation from the transfection procedure outlined in section 4.5.1, aside from the control groups and nucleic acid use, was that the Lipofectamine 3000 treated groups were cultured for 48 hours in transfection media as instructed by the manufacturer, rather than the sequential 24 hour incubations in transfection media and normal media performed on all other groups. Following the MTT protocol outlined in section 4.5.2, cells were rinsed with PBS thrice at 48 hours post-transfection and incubated for 4 hours in serum-free media and TBTB dissolved in PBS. Cells were rinsed three more times in PBS and 1 mL of DMSO added to all wells. The plate was wrapped in foil and placed on an orbital shaker for 10 minutes to allow for complete dissolution of the formazan crystals formed during their incubation with TBTB. DMSO was removed to a 96-well plate and the absorbance at 570 nm measured in triplicate using a Biotek Synergy HT plate reader. Relative cell viability was calculated as Cell Viability (%) = $(OD_{570\ (sample)}/OD_{570\ (control)})*100$

4.7.2 MDR1 Silencing

The degree of MDR1 silencing by PgP-mediated transfection with siMDR1 was quantified in MDA-MB-435 ADR cells. MDA-MB-435 ADR cells were transfected using the same procedure as outlined in 4.7.1, in 24-well plates seeded at $6x10^4$ cells/well and cultured for 24 hours prior to transfection. Polyplexes were formed at N/P ratios of

30:1, 45:1, and 60:1 in sterile nuclease free water and incubated at 37 °C for 30 minutes to allow formation of stable complexes. Control groups included untreated cells and cells treated with Lipofectamine 3000 delivering siMDR1. At 48-hours post-transfection, cells were harvested and isolated for either protein or RNA, to evaluate the MDR1 expression level by western blot in protein level and real-time PCR (RT-PCR) in mRNA level, respectively.

i) Western blot

At 48 hours after transfection, cells were washed with PBS and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% triton-x) containing 0.1% Halt Protease & Phosphatase Inhibitor Cocktail (see 3.1: materials), all chilled to 4 °C prior to addition into the well plate. Wells were scraped using the rubber plunger of a 1 mL syringe, briefly triturated, and removed to Eppendorf tubes which were then placed on ice for 10 minutes to allow complete cell lysis. Samples were briefly vortexed at low speed and then centrifuged at 10,000 rpm and 4 °C for 15 minutes, after which samples were removed to new Eppendorf tubes without disturbing the pellet formed during centrifugation. Concentration of the samples was determined by BCA assay using the Pierce BCA Protein Assay Kit, performed according to the manufacturer's protocol. 10 μg of protein lysates from each sample were then loaded into SDS-Page gels and run for 15 minutes at 150 V, followed by 90 minutes at 120 V once samples had entered the stacking gel. Proteins were then transferred to Immuno-Blot Polyvinylidene fluoride (PVFD) membranes for one hour at 100V. Membranes were then submerged in blocking solution (4% Bovine Serum Albumin

(BSA) in Tris-HCl buffered saline with 0.1% Tween-20 (TBST)), for one hour at room temperature on an orbital shaker at low speed. After blocking, primary antibody binding was accomplished by incubating the membranes in blocking solution containing either mouse monoclonal anti-MDR1 antibody (1:800 dilution) or mouse monoclonal anti-β-actin antibody (1: 1,000) for 16 hours at 4 °C. Membranes were then thrice washed for 5 minutes in TBST and incubated in blocking solution containing horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (1: 8,000) for one hour at room temperature. Membranes were once again washed for 5 minutes in TBST three times followed by a 5-minute incubation at room temperature with 1 mL of SuperSignal West Pico Chemiluminescent Substrate. Membranes were then sealed in plastic wrap and imaged using the ChemiDox-It system with no filter applied and an exposure time of 5 minutes.

ii) **RT-PCR**

At 48 hours post-transfection, cellular RNA was isolated and purified using the RNeasy Mini Plus Kit according to the manufacturer's protocol. Genomic DNA (gDNA) was eliminated using the TURBO DNA-Free Kit, after which the concentration and purity of RNA samples was determined by absorbance at 260, 280, and 320 nm measured with a Take3 Micro-Volume Plate and a Biotek Plate Reader. 10 µg of each RNA sample was then reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit with MultiScribe Reverse Transcriptase according to manufacturer instructions. RT-PCR was performed in duplicate for all samples using the Quantitect SYBR Green PCR Kit, a Rotor Gene Q thermal cycler, and IDT custom forward and

reverse primers for MDR1 and the endogenous control gene GAPDH. A RT-PCR standard curve was generated using RNA samples isolated from untreated MDA-MB-435 ADR cells to determine the amplification efficiency of the target and reference genes, calculated from the slope of the standard curve (C_T vs. log(quantity)) with the equation $e = 10^{-1/slope}$ and found to be within 0.05 of 100% efficient for both genes, with R^2 values of > 0.997. The cycle number at which the fluorescence detected was greater than the threshold line of 0.02, or threshold cycle (C_T), was determined for GAPDH and MDR1 in all samples and relative mRNA expression of MDR1 calculated using the $\Delta\Delta C_T$ method, where $\Delta C_T = C_{T\,(Target)} - C_{T\,(Reference)}$, $\Delta\Delta C_T = \Delta C_{T\,(Sample)} - \Delta C_{T\,(Control)}$, and relative expression (%) = $2^{-\Delta\Delta Ct}$. Controls for all samples run were prepared without reverse transcriptase during reverse transcription (-RT) and used in all RT-PCR experiments as negative controls to identify gDNA contamination, and each RT-PCR experiment included a no-template control (NTC). Melt curve analyses were performed following each run and indicated no formation of primer dimers, nonspecific binding, or other potential sources of error in the procedure.

4.8 Doxorubicin Loading in PgP

To load doxorubicin (DOX) in PgP, the hydrophobic drug DOX was first dissolved in methanol at varying concentrations and added to samples of 1 mg/mL PgP at a 1:10 ratio (v/v), therefore the concentration of DOX in methanol was 10x the final loading concentration used to calculate loading efficiencies and capacities. The samples were shaken for 4 hours at room temperature, and left overnight to allow for methanol

evaporation. After complete evaporation of methanol, sterile deionized water (DW) was added to supplement water loss due to evaporation and the samples were briefly centrifuged. Samples were then filtered using 0.2 μm PES membrane syringe filters.

Absorbance of the samples at 520 nm was measured using a Biotek Synergy HT plate reader, and DOX concentration of each sample was calculated by Beer's law. The standard curve was made using doxorubicin-HCl (DOX-HCl) in deionized water in concentrations ranging from 0.025-0.5 mg/mL and samples read were diluted with a dilution factor of 5 to prevent detector saturation. Entrapment efficiency was calculated as EE=drug loaded (weight)*100/total drug (weight). Loading capacity was calculated as LC (%)=drug loaded (weight)*100/Nanoparticle (weight), used to quantify the drug content of the particle irrespective of its presence in the medium.

4.9 Cytotoxicity of Doxorubicin-loaded PgP

The cytotoxicity of Doxorubicin-loaded PgP (DOX/PgP) was determined by MTT assay performed on MDA-MB-435 ADR cells after treatment with DOX/PgP. Cells were seeded in 24-well plates at a concentration of 6 $x10^4$ cells/well, using a working volume (500 uL) of complete medium and incubated for 24 hours. Cell medium was then replaced with RPMI 1640 cell medium supplemented with 10% FBS and 1% L-Glutamine. DOX/PgP was prepared as outlined in section 4.8 at a loading concentration of 1 mg/mL doxorubicin and diluted with DW to varying concentrations of DOX ranging from 1-25 μM (determined by previously outlined loading study) and a final volume of 50 μL/well. Control groups consisted of untreated cells, cells treated with equivalent

concentrations of DOX-HCl, and cells treated with equivalent concentrations of PgP alone. Cells were then incubated for an additional 24 hours, at which point all wells were rinsed three times with media. Cells were then incubated for 4 hours at 37 °C in 500 µL of serum-free media and 120 µL of 2 mg/mL Thiazolyl Blue Tetrazolium Bromide (TBTB) in PBS. After incubation, cells were rinsed with PBS and 1 mL of DMSO added to all wells, and the plate was wrapped in foil and placed on an orbital shaker for 10 minutes to allow for complete dissolution of the formazan crystals formed during their incubation with TBTB. DMSO was then removed to a 96-well plate and absorbance at 570 nm measured in triplicate using a Biotek Synergy HT plate reader. Relative cell viability was calculated as Cell Viability (%) = $(OD_{570\ (sample)}/OD_{570\ (control)})$*100

4.10 Combinatorial Therapy of Doxorubicin and siMDR1 by PgP

To evaluate the synergistic effect of MDR1 gene knockdown and DOX treatment, we performed 2 different treatment methods, 1) co-delivery of DOX/PgP/siMDR1 and 2) sequential treatment of PgP/siMDR1 and then DOX/PgP.

i) Cytotoxicity of co-delivery of DOX/PgP/siMDR1 in MDA-MB-435 ADR cells

The cytotoxicity of co-delivered DOX and siMDR1 was determined by treatment of MDA-MB-435 ADR cells with DOX-loaded PgP (2.36 µg DOX for N/P ratio of 30:1 and 3.54 µg DOX for N/P ratio of 45:1) complexed with siMDR1 (1 µg siMDR1 for N/P ratio of 30:1 and 45:1). DOX/PgP/siMDR1 complexes were prepared by first loading DOX in PgP according to the procedure outlined in section 4.8 using a DOX loading concentration of 1 mg/mL in PgP (1 mg/mL) and confirmed by absorbance at 520 nm.

DOX/PgP samples were then complexed with siMDR1 at N/P ratios of 30:1 and 45:1 in sterile nuclease-free water and incubated for 30 minutes at 37 °C, prepared immediately prior to addition into the wells. Cells were seeded 24 hours prior to transfection in 24-well plates (6 x10^4 cells/well). Controls for this experiment included untreated cells, and cells treated with PgP alone, DOX-HCl, DOX/PgP, PgP/siNT, PgP/siMDR1, and DOX/PgP/siNT. Cells were incubated for 24 hours, at which point wells were rinsed and replaced with complete media, followed by an additional 48-hour incubation. 72 hours after treatment, cell viability was measured by MTT assay, using the same methods as those outlined in chapter 4.5.2.

ii) Cytotoxicity of sequential treatment of PgP/siMDR1 and DOX/PgP

The cytotoxicity of sequential treatment of DOX and siMDR1 was determined by treatment of MDA-MB-435 ADR cells with PgP/siMDR1 first and then treated with DOX-HCl. PgP/siMDR1 polyplexes (1 µg siMDR1 for N/P ratios of 30:1 and 45:1) was transfected in MDA-MB-435 ADR cells in parallel to the co-delivery study outlined above using the same methods. At 48 hours after transfection, all cells were then treated with DOX-HCl, at an equivalent dose to that of DOX loaded in PgP for the given N/P ratio used (9 µM and 13.5 µM for N/P ratios of 30:1 and 45:1, respectively). After an additional 24-hour incubation, a total of 72 hours after treatment with PgP/siMDR1 polyplexes, cell viability was measured by MTT assay, using the same methods as those outlined in chapter 4.5.2.

4.11 Statistical Analysis

Quantitative data is presented all figures as mean ± SEM. Statistical analyses between two groups were performed with a Student's t-test, and analysis among multiple groups was performed using a one- way analysis of variance (ANOVA). Results were considered statistically significant when $P < \alpha$, where $\alpha = 0.001$ for RT-PCR results, and $\alpha = 0.05$ for all other experiments. All calculations for statistical analysis were performed using the Analysis ToolPak program within Microsoft Excel.

CHAPTER 5

RESULTS

5.1 Transfection Efficiency and Cytotoxicity of PgP/pGFP Polyplexes

Qualitative analysis by fluorescent microscopy showed successful uptake of the particle by the MDA-MB-435 ADR cells, indicated by the expression of GFP which is visible in figure 3. As expected, no fluorescence was visible in cells treated with naked pGFP, having no inherent mechanism to facilitate entry into the cytosol. Cells treated with pGFP/PEI showed very little fluorescence in terms of both intensity and the number of cells exhibiting GFP Fluorescence, due to its poor stability in serum conditions. Of the cells treated with PgP, the N/P 60:1 and 30:1 groups appeared to have similar proportions of cells producing GFP, however the fluorescing cells within the 60:1 treated groups showed a markedly higher intensity of fluorescence than those treated with complexes formed at N/P 30:1. The results, given in figure 4, are consistent with fluorescent imaging, showing very low transfection in PEI and highest transfection in the 60:1 groups and statistical significance between the PgP N/P 30:1 and 60:1 groups in both the wild type and ADR cells at an alpha value of 0.05. Groups treated with naked pGFP showed no significant transfection in comparison to untreated groups, reiterating the need for a delivery system in transfection with pDNA.

Results of the cytotoxicity experiment, given in figure 5, showed minimal toxicity in the groups treated with PgP at N/P ratio of 30:1, with a strong positive correlation between the N/P ratio used and cytotoxicity in all PgP-treated groups. Actual percent

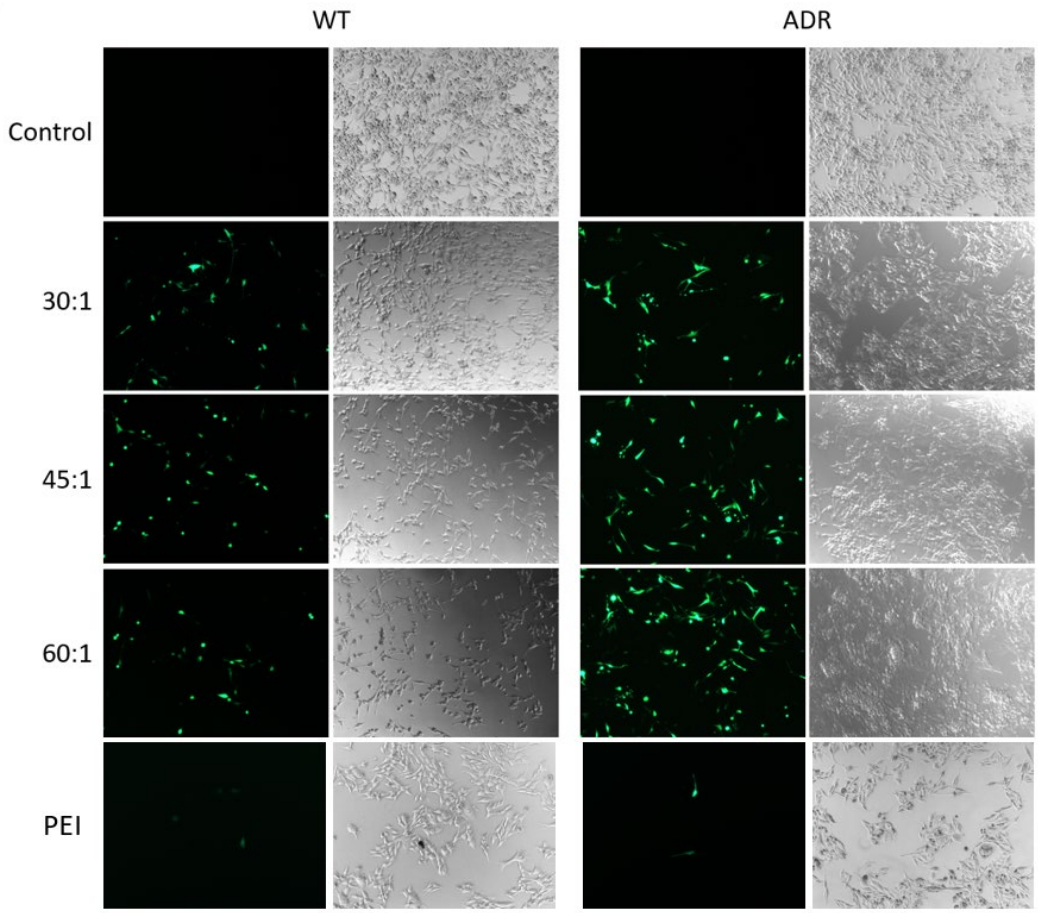

Figure 3. Visualization of transfection by GFP expression in MDA-MB-435 Cells. Both Wild Type (left) and ADR cells (right) were treated with naked pGFP (control), PEI (N/P 7:1) and PgP/pGFP complexed at N/P ratios of 30:1, 45:1, and 60:1. In these images, taken 48 hours after transfection, PgP is shown to successfully mediate delivery of pDNA to the intracellular compartment for release within the cytosol.

viability for all ADR groups relative to the control were 105%, 92%, 77%, 70%, and 90% for PEI 7:1, PgP 30:1, 45:1, and 60:1, and Lipofectamine 3000, respectively. A similar trend was observed in the wild type groups, with cell viabilities of 98%, 80%, 65%, and 45% for PEI 7:1, PgP 30:1, 45:1, and 60:1, respectively. In summary, this study has shown that PgP can be used for delivery of nucleic acid into the cytosol of drug resistant human cancer cells such that they can then function, and that there is potentially an ideal N/P ratio in which transfection efficiency can be maximized while mitigating any

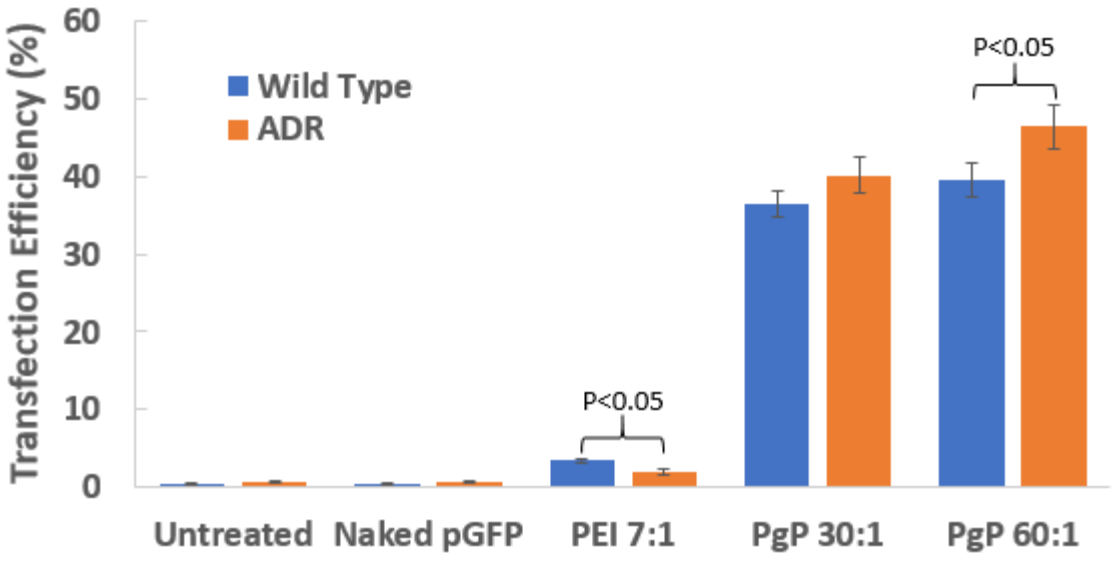

Figure 4. Transfection Efficiency of PgP/pGFP complexes in MDA-MB-435 ADR Cells. Measured by flow cytometry in analysis of GFP expression 48 hours after transfection with naked pDNA, PEI (N/P 7:1), and PgP (N/P 30:1 and 60:1). Data shown is mean ± SEM of three independent experiments (N=3) in which all groups were run in triplicate. Significance was found for all PgP/pGFP groups compared to untreated cells, and significance found between WT and ADR for PEI and PgP/pGFP 60:1 (P<0.05, Student's t-test).

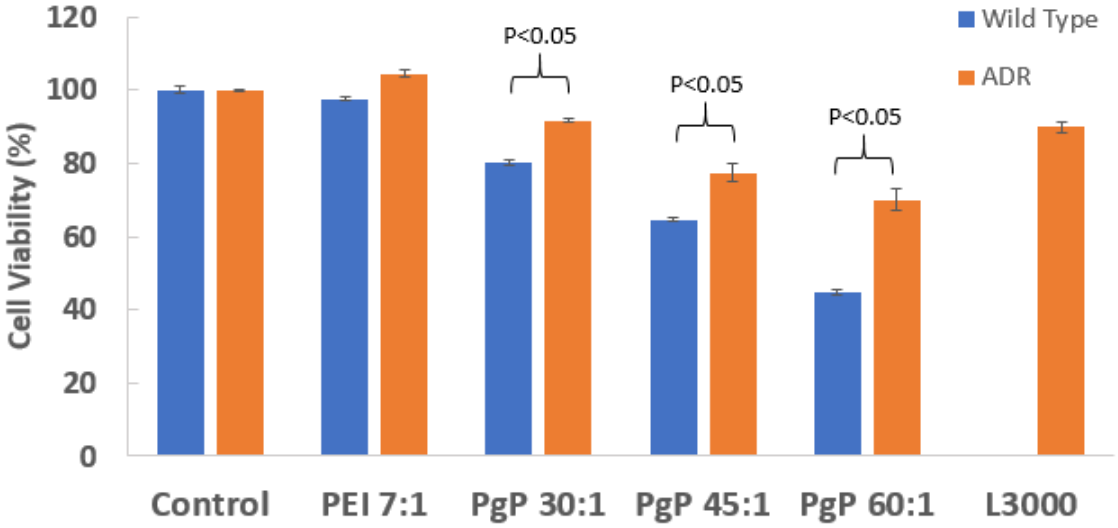

Figure 5. Cell Viability of MDA-MB-435 ADR cells 48 hours after treatment with PgP/pGFP. Relative Cell Viabilities shown were determined via MTT assay and read by absorbance at 570 nm in 96-well plates. Groups shown are (left to right) Untreated cells, PEI/pGFP at N/P ratio 7:1, PgP/pGFP at N/P ratio 30:1, PgP/pGFP at N/P ratio 45:1, PgP/pGFP at N/P ratio 60:1, and pGFP loaded in Lipofectamine 3000. Data is given as mean ± SEM of four independent experiments (N=4) performed in triplicate. Significant difference (P<0.05) was found between WT and ADR cells in all PgP/pGFP groups.

cytotoxic effect of the particle for use as a non-viral vector in therapeutic gene therapy with siRNA.

5.2 Heparin Competition Assay

PgP has previously been shown to effectively mediate transfection in some cell lines with pDNA,[132] however existing research suggests that within the field of synthetic carriers the ability of a vehicle to successfully deliver pDNA may not be as translatable to RNA applications as previously thought.[146] To that end, a heparin competition assay was performed on PgP/pGFP and PgP/siMDR1 polyplexes to assess the binding strength of siRNA relative to that of pDNA. Heparin, due to its strong negative charge, will compete with nucleic acids present in the complex to electrostatically associate with amine groups in the corona of the PgP micelles, ultimately causing nucleic acid to dissociate from the complex when present in high enough concentration. For this study, PgP was complexed with either pGFP or siMDR1 at N/P ratios of 30:1, 45:1, and 60:1 and incubated with varying concentrations of heparin, measured here by their weight ratio (WR) to the nucleic acid present, and compared to naked siMDR1 or pGFP corresponding with the complex used. Imaging of the gels after electrophoresis show that PgP/pGFP complexes consistently begin to dissociate at much higher weight ratios of heparin, indicating a lower competitive stability in siMDR1-containing complexes. Displacement of pGFP can be seen occurring at WRs of 6:1, 9:1, and 12:1 for complexes formed at N/P ratios of 30:1, 45:1, and 60:1, respectively, while in PgP/siMDR1 complexes dissociation is seen much earlier, at WRs of 3:1, 4:1, and 6:1. PgP/pGFP complexes at N/P 30:1. Although

displacement of siMDR1 begins much earlier than pGFP in PgP complexes, partial displacement of siMDR1 can be seen over a much larger range than in pGFP groups. Complete dissociation (as can be referenced by the naked nucleic acid group in each image) of siMDR1 from PgP only occurs after a large range of partial displacement, at weight ratios of 5:1, 9:1, and 10:1 – much closer to those at which pGFP completely dissociates.

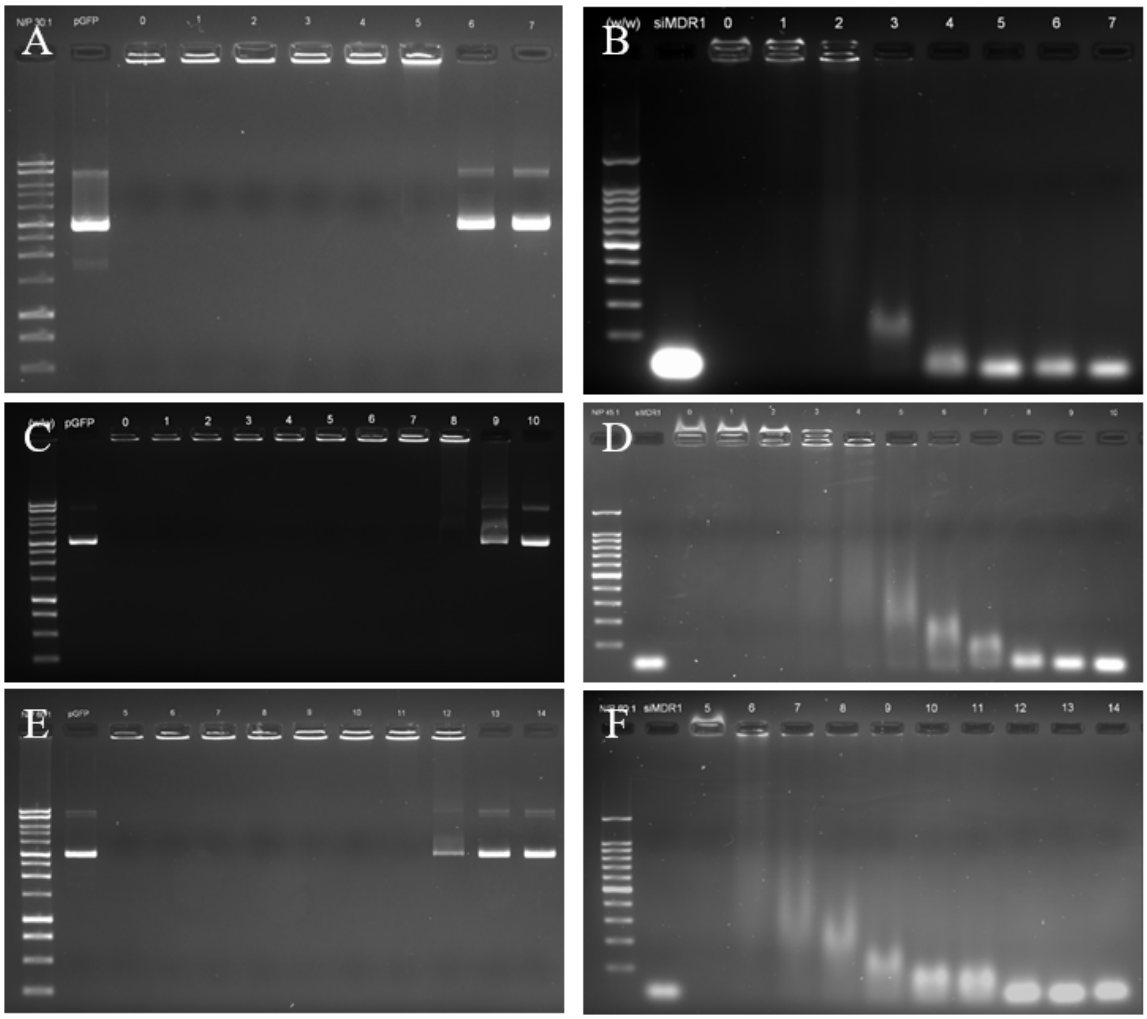

Figure 6. Heparin competition assay. Competitive dissociation of PgP/pDNA in the presence of heparin at varying weight ratios (Heparin/Nucleic acid ratio). Samples shown are (left to right) naked nucleic acid, followed by polyplex samples incubated in increasing ratios (w/w) of heparin to nucleic acid in the sample. A) PgP/pGFP (N/P 30:1) B) PgP/siMDR1 (N/P 30:1) C) PgP/pGFP (N/P 45:1) D) PgP/siMDR1 (N/P 45:1) E) PgP/pGFP (N/P 60:1) F) PgP/siMDR1 (N/P 60:1)

5.3 PgP-Mediated Delivery of siMDR1

In order to assess the ability of PgP to deliver siMDR1 to cells and that the siRNA delivered succeeds in silencing the gene coding for P-glycoprotein (MDR1), MDA-MB-435 ADR cells were transfected with PgP/siMDR1 complexes formed at N/P ratios of

30:1, 45:1, and 60:1, and examined for silencing of MDR1 at the mRNA and protein levels as well as the cytotoxicity of the doses used to produce samples for these knockdown experiments. To assess MDR1 expression on the protein level, the protein levels of each group determined by BCA assay, and each sample analyzed by western blotting for MDR1 expression with β-actin chosen as an endogenous control for normalizing protein level per lane (MDR1 ~170 kDa, β-actin ~42 kDa). Visually, MDR1 protein expression (shown in figure 7) in the control band appears much higher than in the knockdown groups, and when normalized the relative expression of MDR1 was found to be at 52%, 23%, 21%, and 29% when compared to the control for PgP 30:1, 45:1, 60:1, and Lipofectamine 3000, respectively. Silencing of MDR1 at the mRNA level was assessed by RT-PCR, and results given in figure 8. The expression of mMDR1 was normalized using GAPDH as an endogenous control and within 0.5 C_t of the GAPDH C_T value found of the control group. All groups showed significant knockdown in comparison to untreated cells, with PgP complexed at N/P ratios of 30:1, 45:1 and 60:1 showing relative gene expressions of 76%, 65%, and 33%, respectively. Cytotoxicity of these groups was assessed via MTT assay, the results of which are shown in figure 9. In consideration of both the silencing and cytotoxicity results, N/P 45:1 PgP was selected to move forward with in future studies.

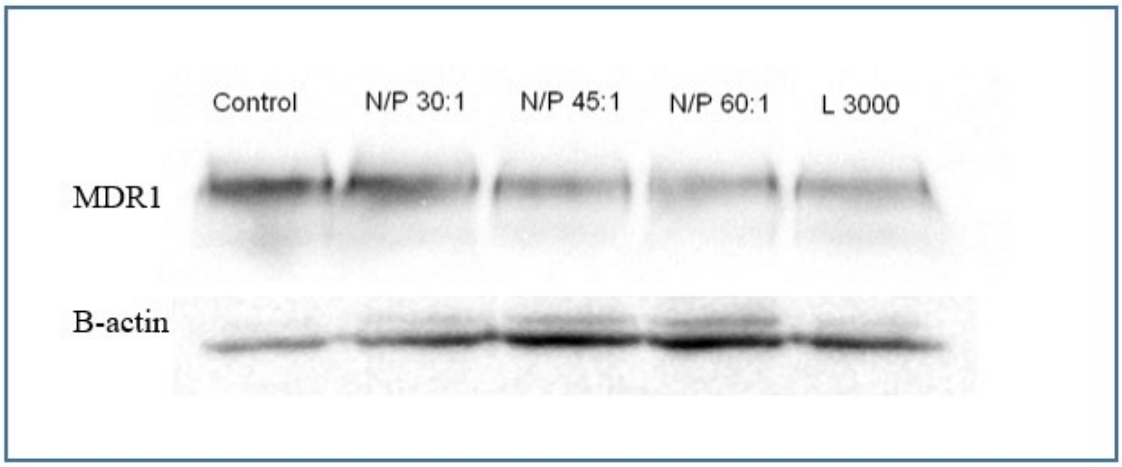

Figure 7. Western blot analysis of MDR1 expression following treatment by PgP/siMDR1. Results reflect MDR1 protein levels in MDA-MB-435 ADR cells at 48 hours after treatment with siMDR1 containing complexes of PgP prepared at N/P ratios of 30:1, 45:1, and 60:1, with untreated cells as the negative control (left) and Lipofectamine 3000 used as a positive control (right). B-actin was used to normalize results for densitometric analysis

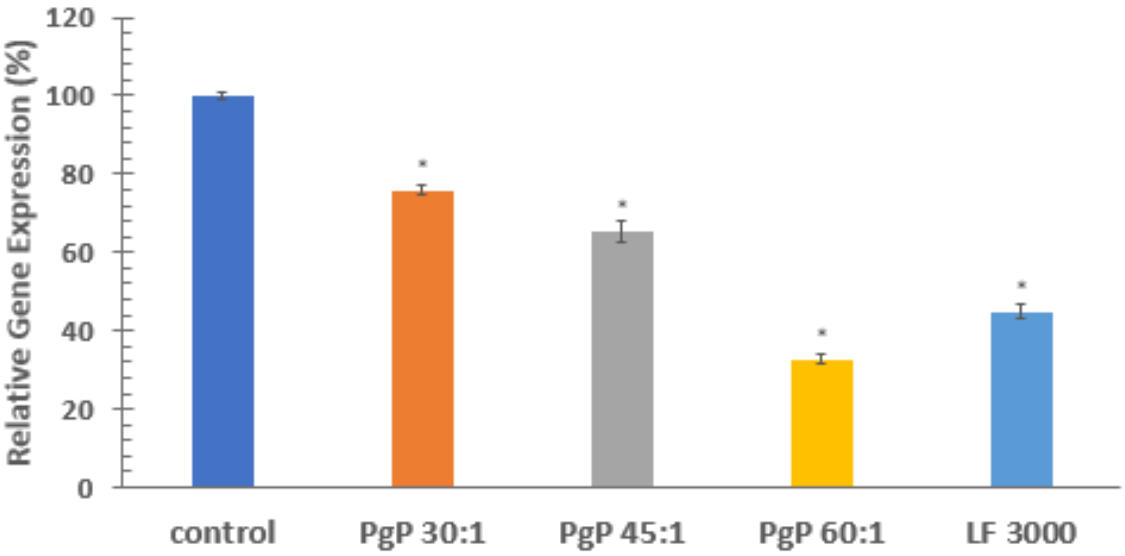

Figure 8. Relative expression of MRD1 after knockdown by PgP/siMDR1 polyplexes by RT-PCR. Results of RT-PCR analysis of MDA-MB-435 ADR cells harvested 48 hours after treatment. Samples shown were (from left to right) untreated, treated with PgP/siMDR1 polyplexes complexed at N/P ratios of 30:1, 45:1, and 60:1, and siMDR1 loaded in Lipofectamine 3000. GAPDH was used as an endogenous control. Data is given as mean ± SEM of four independent experiments performed in triplicate where *P<0.0001, (student's t-test) in comparison to untreated cells.

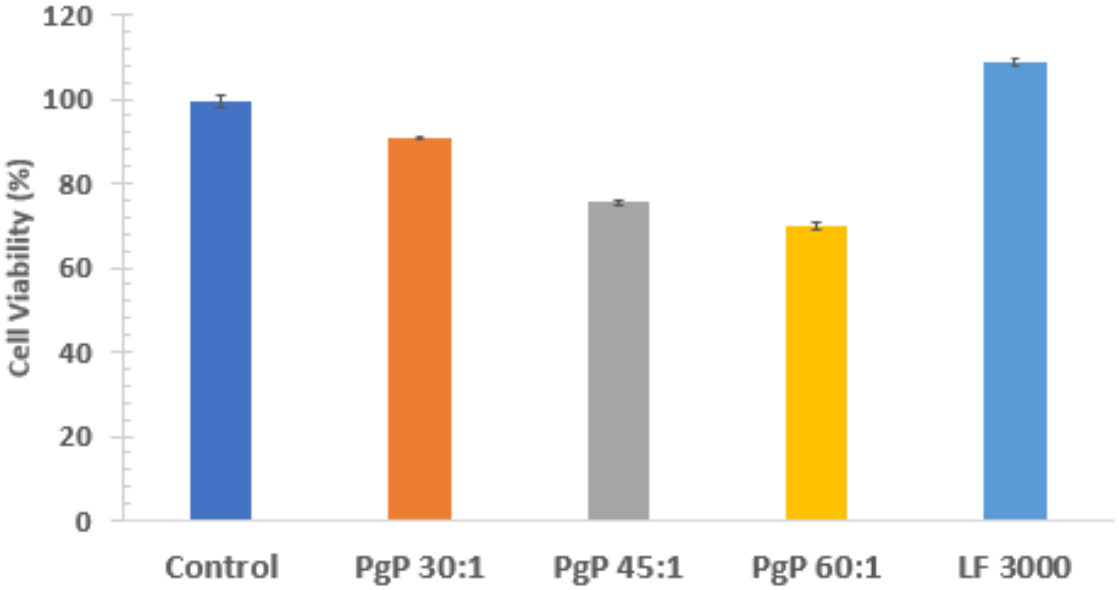

Figure 9. Cytotoxicity of MDA-MB-435 ADR Cells After Treatment with PgP/siMDR1. Relative cell viability was determined by MTT assay with untreated cells, cells treated with PgP/siMDR1 complexed at N/P ratios of 30:1, 45:1, and 60:1, as well as siMDR1 loaded into Lipofectamine 3000. Data shown is mean ± SEM of three independent experiments performed in triplicate. Results were found to be significant ($P<0.05$) by one-way ANOVA.

5.4 Doxorubicin Loading in PgP

Based on the intercalating mechanism through which it functions, a means of intracellularly delivery can significantly improve the efficacy of Doxorubicin. Given the established ability of PgP to deliver pDNA into the cytosol of cancer cells, the capacity of PgP as a carrier for DOX was examined. As shown in figure 10, the ability of PgP to entrap doxorubicin, given by loading capacity, showed a clearly defined positive correlation with the total amount added. However, the entrapment efficiency declined significantly as a result. No steep decline in entrapment efficiency was observed for the concentrations of DOX used that would indicate an approaching maximum capacity of PgP, however the amount of DOX that could be loaded in this analysis was constrained

by the solubility limit of DOX in methanol used in the loading process. Given these results, PgP has shown it can be successfully loaded with DOX, and based on the encapsulation efficiency of DOX/PgP as well as the amount of PgP required for effective transfection, an initial DOX concentration of 1 mg/mL was selected as the loading parameter for further studies with PgP/DOX.

Cytotoxicity of DOX-loaded PgP was determined by MTT assay, in comparison to untreated cells, as well as cells treated with PgP alone or DOX-HCl. As shown in figure 10, a clear increase in cytotoxicity is observed in DOX/PgP treated groups, however the relative cell viabilities of the groups tested leave it unclear if this increase is due to any synergistic effect of the PgP such as an improved efficacy due to intracellular delivery of DOX.

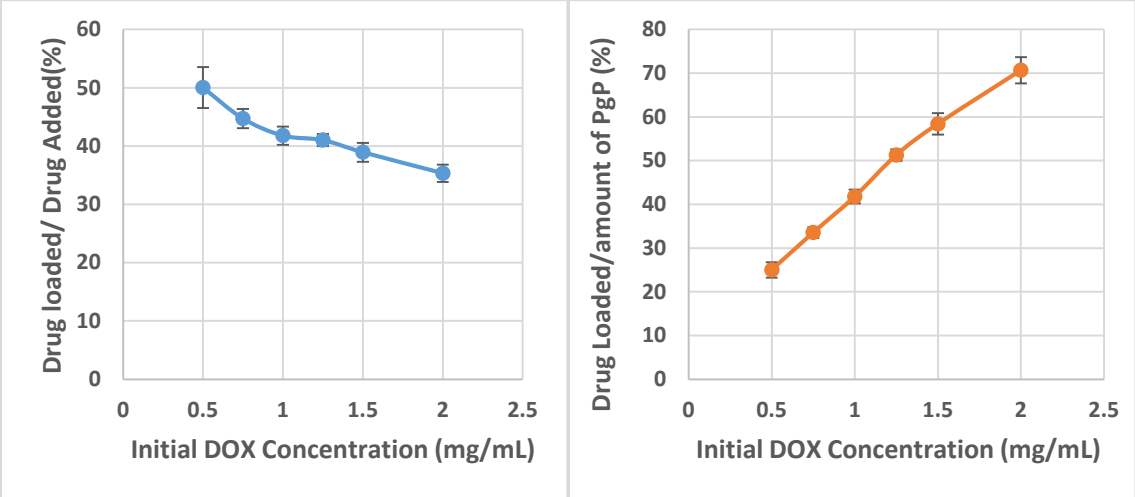

Figure 10. Doxorubicin Loading in PgP. (Left) Entrapment Efficiency of Doxorubicin in PgP, calculated as the amount of drug loaded divided by the total drug added (Right) Loading Capacity of Doxorubicin in PgP, the actual ability of the particle to entrap DOX, and calculated as the Loading= loaded drug (weight)*100/PgP (weight). Data is given as mean ± SEM of three independent studies performed in triplicate.

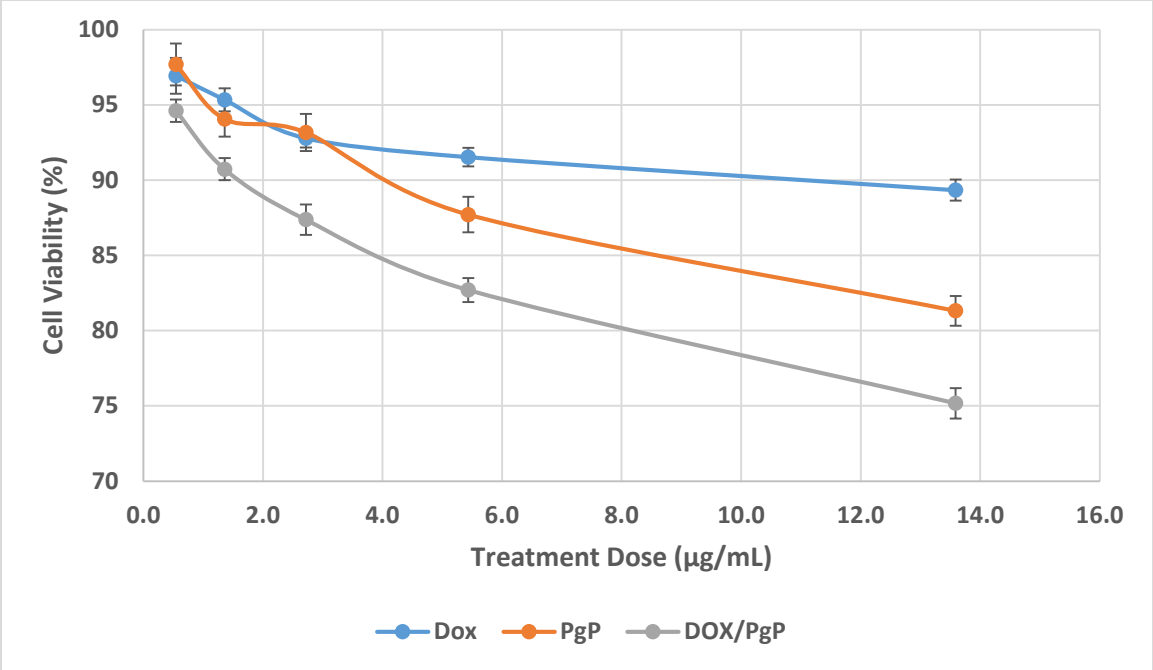

Figure 11. Cytotoxicity of DOX-Loaded PgP Micelles. Doxorubicin was loaded into PgP micelles with an initial concentration of 1 mg/mL and loading efficiency determined at 42%. This value was used in calculating equivalent dose of DOX-HCl, taking into account the difference in molecular weight between DOX freebase and the HCl formulation. PgP dose used was equal to the dose of DOX-loaded PgP. Results are given as mean ± SEM of three independent experiments performed in triplicate.

5.5 Combinatorial Therapy of Doxorubicin and siMDR1 by PgP

To determine the practical efficacy of the fully assembled DOX/PgP/siMDR1 particle, Cytotoxicity studies were performed using MTT assays of MDA-MB-435 ADR cells treated both concurrently and sequentially with PgP/siMDR1 and Doxorubicin, using complexes prepared at N/P 45:1. For assessing the ability of co-delivered siMDR1 and DOX to treat drug resistant cells, groups were treated with DOX-HCl alone, PgP and DOX-loaded PgP complexed with siMDR1, and formulations of PgP or DOX-loaded PgP either alone or complexed with non-targeting siRNA (siNT). As seen in Figure 12, all groups treated with doxorubicin loaded PgP showed significant cytotoxicity over other

groups, with a slight decrease in cytotoxicity observed in the DOX/PgP/siNT group compared to uncomplexed DOX/PgP. A significant increase in cytotoxicity was seen in the DOX/PgP/siMDR1 groups in comparison to non-targeting groups (DOX/PgP/siNT), however the greatest contributor of toxicity to treated groups appears to be doxorubicin delivery by PgP.

In sequentially treated groups, treated with DOX/PgP (13.5 μM DOX) 48 hours after an initial transfection with PgP/siRNA, cytotoxicity was determined by MTT assay, the results of which are shown in figure 13. As with the co-delivery study, significantly higher cytotoxicity was observed in the PgP/siMDR1 groups compared to non-targeting (PgP/siNT) groups, however once again treatment with DOX/PgP seems to be the greatest contributor to toxicity even in this drug-resistant cell line. Given the static nature of the *in vitro* environment as well as the inherently time-dependent nature of siRNA-mediated gene silencing, *in vivo* studies may be necessary to determine the optimal time frame of re-sensitization in cancer populations. Despite this, even the results of short-term studies have demonstrated the ability of PgP in mediating both MDR1 knockdown as well as intracellularly delivering doxorubicin, establishing its capacity as a delivery vehicle for both DOX and siRNA, and justifying its potential candidacy for further characterization *in vivo.*

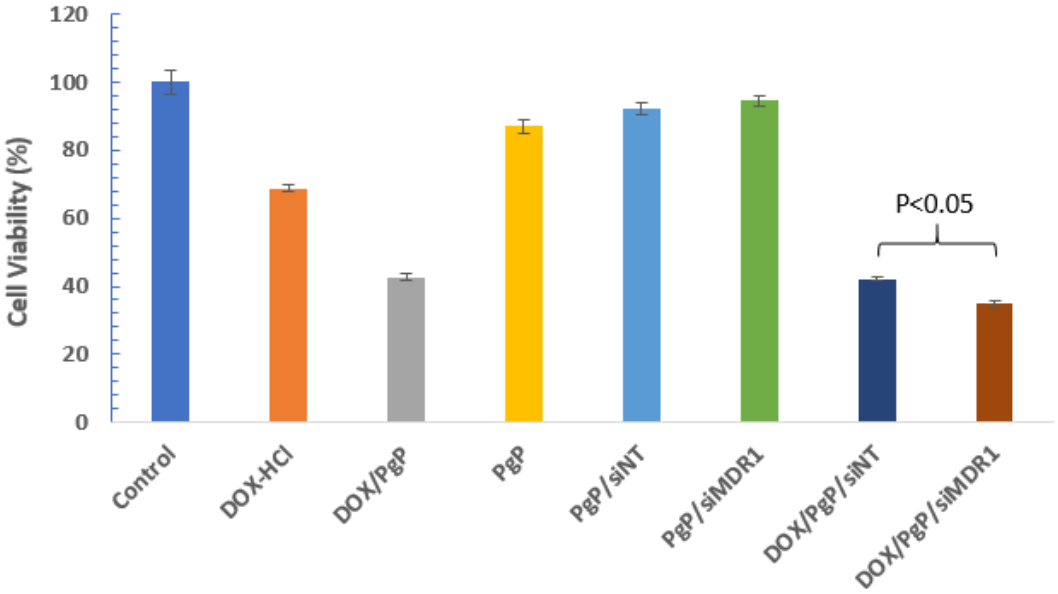

Figure 12. Cytotoxicity of PgP mediating Co-delivery of doxorubicin and siMDR1. MDA-MB-435 ADR cells were treated with the given groups, with all PgP doses reflecting complexation at a N/P ratio of 45:1. All groups were analyzed at 48-hours post treatment via MTT assay. Data is shown as mean ± SEM, with significance found between DOX/PgP/siNT and DOX/PgP/siMDR1 groups ($P<0.05$) by t-test.

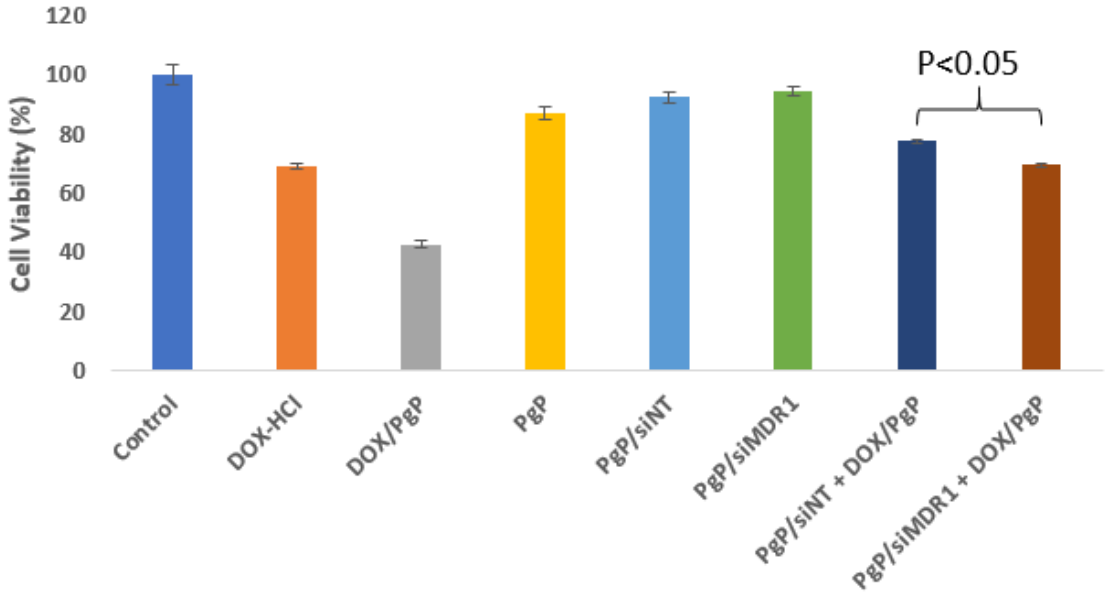

Figure 13. Cytotoxicity of sequential treatment with PgP/siMDR1 and Doxorubicin HCl. MDA-MB-435 ADR cells were treated with the shown groups, and 48 hours later groups indicated by '+DOX-HCl' were given a second treatment of 13.5 µM Doxorubicin HCl. Cytotoxicity of all groups was then assessed by MTT assay at 72 hours after initial treatment. Data is given as mean ± SEM. Significance was found between PgP/siNT and PgP/siMDR1 groups given a secondary treatment of DOX/PgP by t-test ($P<0.05$).

CHAPTER 6

DISCUSSION

The goal of this project was to develop and characterize the novel polymeric micelle developed by this lab, PgP, for its application and utility in mitigating multidrug resistance in metastatic breast cancer. Establishing the safety and utility of its use in both doxorubicin and siMDR1 delivery could prove relevant in treatment of triple-negative breast cancer, such that it could improve the efficacy of normal treatment with chemotherapeutics of the anthracycline class characterized by their hydrophobicity and intercalating mechanism of function. Improving the efficacy of these drugs would result in a lower minimal therapeutic dose and decrease both the risks of systemic cytotoxicity they present as well as the rate at which drug resistance is developed in treated cancer populations due to the inherently cyclic nature of treatment with these drugs. Ultimately should these improvements be translated to clinical application they could serve to improve treatment in terms of both safety and patient outcomes.

In evaluating the results of the experiments listed in this study, a few advantages and shortcomings in the use of PgP as a delivery vehicle for doxorubicin and siRNA that should be addressed. PgP was first examined for its ability to deliver pDNA in such a way that it could then be functionally expressed by transfected cells. Results of these tests proved PgP to be an effective vector for this application, however the trade-off between maximizing particle efficacy and minimizing treatment toxicity is immediately apparent. While increases of dose and N/P ratio of the polyplex did show a positive correlation to

transfection efficiency in these experiments, the increase was not as linearly correlated as could be expected – likely a result of the increase in cytotoxicity arising from the increase in both dose and particle surface charge. Transfection efficiency of complexes formed with pDNA was also observed to be lower than initially hoped, however not to a degree that would eliminate PgP as a promising candidate for siRNA delivery. While a vector's ability to transfect cells with pDNA has been a widely used method of determining its capacity for use in RNAi applications, pDNA and siRNA have been shown to behave very differently when interacting with cationic DDVs, as well as their complex's subsequent interactions in the biological environment.[146] The implications that this difference in behavior between pDNA and siRNA complexed particles could have for this project's goals with PgP as a delivery vehicle were unclear, however, as the nature and significance of this difference seems largely dependent on the particle being examined and its individual charge and complexation to nucleic acid. With that in mind, the binding strength of PgP to siRNA relative to that of pGFP was examined at various N/P ratios through heparin competition, with surprising results; showing stable binding at significantly higher heparin concentrations in the pGFP-bound micelles, while exhibiting similar points of complete dissociation.

Heparin competition revealed siRNA-bound complexes to have a lower overall binding strength to PgP than pGFP-containing polyplexes, although dissociation of nucleic acids within the complex through competition with other charged particles in solution occurred over a much larger range of concentrations than the pGFP-bound groups, which showed a nearly binary relationship when it comes to competition-driven

dissociation with exception to complexes formed at very high N/P ratios. This would imply that PgP/siRNA complexes may show the same drastic increase in efficacy at high N/P ratios for knockdown that the PgP/pDNA complexes did in transfecting GFP expression, however, as their mechanisms of function within the cytosol differ, it is unclear to what degree the actual amount of siRNA delivered to a cell may impact target gene expression.

siMDR1 complexes consistently showed dissociation at much lower weight ratios of heparin, despite the fact that nucleic acid loading was controlled for all samples by mass, making the overall charge of the siRNA added slightly stronger than that of the pDNA due to the lower molecular weight of RNA base pairs. Additionally, the results of the heparin competition would indicate that despite the significantly smaller size of the siRNA, it primarily binds to the surface of the micelle in the same manner as pDNA, rather than interpenetrating the PEI layer of the PgP micelles to associate with deeper amine groups, although confirmation of this through AFM or SEM would be necessary to confirm this indication.

This test was followed by in depth assessment of MDR1 knockdown by PgP/siMDR1 treated groups. RT-PCR and western blot testing both showed significant silencing of the MDR1 gene on both a mRNA and protein level at 48 hours after treatment, to levels much more significant than those given by pDNA transfection studies. While unexpected, this is not unprecedented as PEI and its derivatives have shown in previous studies (albeit unreliably) for improved performance as a vehicle for siRNA over pDNA.[146]

Having established the ability of PgP to mediate silencing of the gene coding for P-glycoprotein (MDR1), the capacity of PgP as a carrier for the hydrophobic drug doxorubicin was then examined. PgP was quickly determined to be capable of loading doxorubicin, although its release over time in serum conditions would be an interesting relationship to quantify, as its stability in PgP micelles in water alone would not be representative of its behavior *in vivo* due to the relative insolubility of doxorubicin in water and the abundance of hydrophobic compounds capable of acting as a solvent to the drug in biological conditions. The loading capacity and entrapment efficiency of doxorubicin in PgP showed normal behavior, although this relationship is likely dependent on the formulation of PgP apropos its PLGA chain length. With the 12 kDa PLGA chain present in the formulation of PgP used in this study, the maximum loading capacity of PgP was limited by the solubility of doxorubicin in the methanol used in the loading process rather than the actual solubility of doxorubicin in PgP. This could be overcome through the use of alternative solvents in loading, however based on the results found and the potential for PgP degradation by stronger organic solvents, the model used was found to be acceptable for this study. In consideration of PgP toxicity required dose, as well as the loading data with regards to the cost and dosage of doxorubicin in clinical applications, loading conditions of 1 mg/mL initial doxorubicin and PgP concentrations were selected for all subsequent studies involving DOX-loaded PgP. The relative cytotoxicity of DOX/PgP in comparison to DOX-HCl and PgP alone showed that delivery mediated by PgP micelles improved efficacy of the drug over the salt formulation, although at high doses beyond what would realistically be used for PgP

showed that it is likely the primary source of toxicity, as seen by the parallel decrease in cell viability exhibited by PgP and DOX/PgP groups that was not reflected in similarly increasing doses of DOX-HCl. To confirm the actual mechanism of toxicity in dox-loaded PgP, whether a function of PgP exposure, its combination with doxorubicin, or due to intracellular delivery of DOX, a TUNEL assay may be incorporated into future studies. Since the function of DOX, like other anthracyclines, produces double stranded DNA breaks in affected cells,[147] TUNEL may be useful in differentiating which of the possible factors contribute most heavily to the observed increase in cytotoxicity by DOX/PgP treatments.

The results of the *in vitro* studies would, at this point, merit progression to *in vivo* characterization of its biocompatibility and antitumor effects, however both time and fiscal restraints dictated further *in vitro* examination into the practical advantages of co-delivering siMDR1 and doxorubicin. While the DOX/PgP/siMDR1 treated groups showed radically lower cell viability than those treated with equivalent doses of DOX-HCl, this was likewise reflected in both the non-targeting groups (DOX/PgP/siNT) and the unbound DOX/PgP groups, prompting us to consider the time-dependent nature of RNAi-mediated gene silencing. The effects of sequential treatment with PgP/siMDR1 followed by doxorubicin-HCl was examined, and these results did show significant cytotoxicity of the siMDR1-treated cells in comparison with non-targeting groups, however not to the scale or degree expected given the knockdown results. While this could indicate the need to address another mechanism of resistance, such as bcl-2 dependent resistance, with siRNA in conjunction to the multidrug resistance mediated by

P-glycoprotein, it may simply represent the limitations of *in vitro* analysis of the particle at this stage. Given the static nature of the *in vitro* studies available to this lab, a more translatable model may be needed. Additionally, determining the time frame and relevant toxicities of optimum treatment may require a more complex model when considering the time frame of transfection and siRNA-mediated gene silencing. While mRNA levels will decrease within 24 hours of siRNA-mediated gene silencing, maximum silencing will often be reached at a later point, usually sometime between 24 and 48 hours following transfection. Functional protein levels, being further downstream, will take even longer to reflect changes in RNA expression and are heavily dependent on the nature of the protein in question and its stability within the experimental system.

Certainly, based on the ability of PgP/siMDR1 complexes to facilitate knockdown of MDR1, as well as its ability to intracellularly delivery doxorubicin, PgP shows potential as a vehicle for mediating multidrug resistance in cancer. Following *in vivo* characterization, modifying the siRNA target to alternative or additional genes responsible for drug resistance such as those in the bcl-2 resistance pathway, or further downstream the pump-dependent (MDR1) pathway may serve to improve treatment efficacy. Including siRNA targeting genes responsible for other proteins essential in metastatic site development such as VEGF or HIF-1α (critical in angiogenesis and directing metastatic proliferation) could also serve to impede proliferation of non-transfected cells within the tumor or pre-tumor environment. Another potential modification to the vector that could be investigated is conjugation to a targeting moiety. Currently, PgP micelles remain a 1st generation nanoparticle in that they contain no

mechanism for specificity beyond passive targeting via the EPR effect. Potential mechanism for targeting could include conjugation to growth factors matched to upregulated receptors in target cancer populations (2nd gen.), or even conjugation to specific antibodies targeting cancer cells (3rd gen.). One potential setback to consider in this modification would be the proximity of a given targeting moiety to the charged PEI corona of the micelles, therefore potential implementation of a hydrophilic spacer should be considered as well – such as polyethylene glycol or other similar groups. Lastly, complexation with siRNA supramolecular assemblies could also prove to act synergistically with their previously established improvements on traditional siRNA in terms of functionality,[148] although this is purely speculative and would require additional characterization of the complex, as incorporation of these structures may impact the polyplex morphology, charge, or behavior in unforeseen ways as a result of their size and flexibility, similarly to the differences in behavior observed in pDNA and siRNA-bound complexes.

CHAPTER 7

CONCLUSIONS

In conclusion, PgP has been definitively proven to effectively complex with siRNA and mediate successful delivery into the cytosol of MDA-MB-435 ADR cells. In comparison to PEI it has shown to be drastically more effective in serum conditions, making it much more relevant to therapeutic applications. While moderate cytotoxicity has been observed in higher N/P ratio complexes, those formed at N/P 45:1 and 30:1 show acceptably low cytotoxicity to justify further assessment *in vivo.* Moreover, PgP/siMDR1 complexes have been shown to significantly reduce expression of MDR1 at the mRNA level *in vitro*, with reductions as high as 67% observed in the N/P 60:1 complexes. In terms of its ability to deliver doxorubicin, PgP has shown to be an effective carrier of the drug and even improve efficiency of the drug in clinically relevant doses. The process of loading is simple and rapid, and compatible with 0.2-micron filter purification.

Overall, PgP has shown to be a promising vector for siRNA delivery in drug resistant cancer cells, and further development in terms of its conjugation with a targeting moiety may result in a clinically relevant vector for not only treatment of multidrug resistance, but a range of applications within the field of gene therapy.

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